Transcriptional activation and sensing properties of DegS-DegU: a two-component system involved in the osmotic regulation of *Bacillus subtilis*

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The voyage of discovery is not in seeking new landscapes but in having new eyes

- Marcel Proust

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Abbreviations

ADAM	1-aminoadamantam
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AHT	Anhydrotetracycline
Amp^r	Resistance towards ampicilin
aphA3, Kan^r	Resistance towards kanamycin
APS	Ammoniumperoxodisulfate
bp	Base pairs
BCA	Bicinchoninic acid
cat, Cml^r	Resistance towards cholramphenicol
CIAP	Calf intestine alkaline phosphatase
DEPC	Diethyl Pyrocarbonat
DIG	Digoxygenin
DTT	Dithiothreitol
EDTA	Ethylendiaminetetraacetic acid
Erm, Ery^r	Resistance towards erythromycin
et. al.	et alii (“and others”)
FMOC	9-Fluorenyloxycarbonylchlorid
g	Gravitational acceleration (9.81m/s)
HPLC	High-performance liquid chromatography
IPTG	Isopropyl-1-thio-β-D-galactosid
kDa	kilo Dalton
LB	Luria-Bertani
MM	Minimal medium
MOPS	3-[N-morpholino] propanesulfonic acid
OD_{578}	Optical densitiy at 578nm
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PNPG	*para*-Nitrophenyl-α-D-Glucopyranoside
Rpm	Rotations per minute
RT	Room temperature
SDS	Sodium dodecylsulphate
TAE	Tris acetate EDTA
TEMED	N,N,N’,N’-tetramethyl-ethylendiamine
Tris	2-aminohydrohymethylpropane-1,3-diol
v/v	Volume per volume
w/v	Weight per volume

I. Summary

Water availability is crucial for the development of all living cells. Various physical and chemical parameters such as desiccation and hyperosmotic stress generate cellular dehydration. The mechanisms of cellular adaptation preventing water loss under hyperosmotic conditions (osmoregulation) have been extensively studied in many organisms. In *Bacillus subtilis* the DegS-DegU two-component regulatory system controls various processes that characterize the transition form the exponential to the stationary growth phase, including the induction of extracellular degradative enzymes, expression of late competence genes and down regulation of the sigma D regulon, which encompasses the genes involved in motility, chemotaxis and autolysin production. Besides, the system is the only one up to now that is expressed under hyperosmotic conditions (Steil et al., 2003). In this work the role of the DegS-DegU system was further investigated with respect to its role upon salt induction.

Northern blot experiments performed together with reporter-gene fusion studies confirmed that both *degS* and *degU* genes are transcribed together as an operon. Additionally, the presence of internal promoter upstream from the *degU* gene could be demonstrated. Involvement of the DegS-DegU system in the osmotic regulation was supported by the accumulation of mRNA transcripts from both genes when the cells were subjected to hypertonic conditions. The detected internal *degU* promoter was shown to be activated through a positive feedback mechanism from the phosphorylated DegU protein. Investigations of the main *degS-degU* promoter in the presence of different charged and non-charged compound, which raised the surrounding osmolarity to an equal extent, demonstrated that the activation of the system is rather an osmotic response and not consequence of salt-specific stimulation. Moreover, it seems that the DegS-DegU system does not sense the osmolarity per se since the addition of the potent osmoprotectant glycine betaine did not exhibit any influence and the corresponding structural genes were still induced.

The cytoplasmic localisation of the DegS protein raised the following question: what are the molecular mechanisms underlying the perceiption of a certain stimuli from the environment and their transition to the sensor kinase. For this purpose both proteins have been purified and *in vitro* experiments were established for the detailed analysis of the DegS sensing properties. The *in vitro* investigations of the DegS autokinase activity in the presence of a variety of osmolytes with different chemical nature showed that the sensor kinase was specifically stimulated by the presence of glutamate. The putative role of the latter in activating the DegS

autophosphorylation was supported also from the fact that osmotically challenged cells accumulated glutamate immediately after the addition of NaCl to the growth medium.

In parallel to exploring the role of the DegS-DegU system at the transcriptional and at the protein level, some additional experiments were performed in order to identify possible downstream regulated targets of the system. The utilization of wild type (*degS degU*), hyper mutant [*degU32*(Hy)] and deletion mutant (*degS degU*::aphA3) strains led to the identification of nine genes whose transcription was increased when the cell were subjected to high osmotic environment and concomitantly this expression was dependent on the availability of *degS* and *degU* gene products.

Taken together, the data from the present work shed a light on the transcriptional regulation of DegS-DegU two-component system in *B. subtilis* and implicated intracellular glutamate as a positive stimulus involved as a transducer of the information from the environment to the intracellular apparatus.

Zusammenfassung

Wasserverfügbarkeit ist wichtig für die Entwicklung lebender Zellen. Verschiedene physikalische und chemische Parameter, wie niedrige Wasserverfügbarkeit und hyperosmotischer Stress führen zu zellulärer Dehydration. Die Mechanismen der zellulären Anpassung zur Vermeidung von Wasserverlust unter hyperosmotischen Bedingungen (Osmoregulation) sind in vielen Organismen intensiv untersucht worden. In *Bacillus subtilis* kontrolliert das Zwe-Komponenten System DegS-DegU verschiedenen Prozesse, welche charakteristisch für den Übergang von der exponentiellen zur stationieren Wachstumsphase sind. Dies sind unter anderem die Induktion extrazellulärer Abbauenzyme, die Expression von Genen für die Kompetenz, oder die negative Regulation des Sigma D Regulons, welches die Gene für die Bewegung, die Chemotaxis und die Autolysinproduktion umfasst. Das DegS-DegU System ist bislang das einzig bekannte, welches unter hyperosmotischen Bedingungen expremiert wird (Steil *et al*., 2003). In dieser Arbeit wird die Rolle dieses regulatorischen Zwei-Komponentensystems dahingehend untersucht, welche Rolle die Aktivierung durch Salz spielt.

Northern-Blot Experimente und Daten aus Reportergenfusionen bestätigen, dass beide Gene, *degS* und *degU,* gemeinsam als Operon transkribiert werden. Zusätzlich existiert noch ein interner Promoter stromaufwärts von *degU*. Die Beteiligung des DegS-DegU Systems an der osmotischen Regulation wurde unterstütz durch die Akkumulation der mRNA von beiden Genen, wenn die Zellen unter hyperosmotischen Bedingungen kultiviert wurden. Für den internen *degU* Promoter konnte eine Aktivierung durch einen positiven Feedback-Mechanismus duerch das phosphorylierte DegU-Protein nachgewiesen werden. Untersuchungen des *degSU* Promoters in der Gegenwart verschiedener geladener und ungeladener Substanzen unter vergleichbaren osmotischen Verhältnissen zeigten, dass die Aktivierung des Systems ein osmotischer, und kein salzspezifischer Effekt ist. Außerdem scheint das DegS-DegU System nicht per se auf die Osmolarität zu reagieren, da die Zugabe des potenten Osmoprotektivums Glycin-Betain keinen Einfluss hat und die korrespondieren Gene weiterhin induziert bleiben.

Die zytoplasmatische Lokalisation von DegS wirft folgende Frage auf: was sind die molekularen Mechanismen, die der Wahrnehmung eines Sidnals aus der Umwelt und ihrer Weterleitung zur Sensorkinase zugrunde liegen. Um dieser Frage nachzugehen, wurden beide

ZUSAMMENFASSUNG

Proteine chromatographisch gereinigt und *in vitro* Experimente etabliert, um eine detaillierte Analyse der DegS Eigenschaften durchzuführen.

Die *in vitro* Unrtersuchungen der DegS Autokinase-Aktivität in Gegenwart einer Auswahl von Osmolyten mit unterschiedlichen chemischen Eigenschaften zeigten, dass die Sensorkinase nur in Gegenwart von Glutamat spezifisch stimuliert wird. Eine mögliche Rolle bei der Aktivierung der DegS Autophosphorylierung wurde auch durch die Tatsache unterstütz, dass Zellen nach osmotischen Veränderungen Glutamat direkt nach der Zugabe von NaCl zum Wachstumsmedium akkumulieren.

Parallel wurde die Rolle des DegS-DegU Systems auf transkriptioneller und auf Proteinebene untersucht. Zur Identifikation möglicher weiterer, durch dieses System regulierter Gene, wurden zusätzliche Experimente durchgeführt. Die Verwendung des Wildtypstammes (*degS degU*) einer Hypermutante (*degU32*(Hy)) und einer Deletionsmutante (*degS degU*::*aphA3*) führten zur Identifizierung von neun Genen deren Transkription ansteigt, wenn die Zellen einer Umgebung mit hoher Osmolarität ausgesetzt werden. Die Expression dieser Gene ist außerdem abhängig von der Verfügbarkeit der *degS* und *degU* Genprodukte.

Die Daten dieser Arbeit geben einen Einblick in die transkriptionelle Regulation von und durch das DegS-DegU Zweikomponrentensystem in *B. subtilis* und zeigen intrazelluläres Glutamat als einen positiven Stimulus bei der Weiterleitung von Informationen aus der Umwelt in das Zellinnere.

II. Introduction

As part of their surrounding environment, all living organisms, including pro- and eukaryotes, are exposed to the biological, chemical and physical parameters defining their habitat. In order to survive, they have to be able to sense changes in the environmental parameters and react to them by using various mechanisms of adaptation. Understanding the strategies that are used for coping with such stress conditions is important for both basic microbiology and applied biochemistry.

1. *Bacillus subtilis*

Bacillus subtilis is the best-characterized member of the Gram-positive bacteria. It is a facultatively anaerobic, rod-shaped bacterium commonly recovered from water, soil, air and decomposing plant residue. It has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. The endospore is formed at times of nutritional stress, allowing the organism to persist in the environment until conditions become favorable. Prior to the decision to produce the spore the bacterium might become motile, through the production of flagella, and also take up DNA from the environment. *B. subtilis* produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. *B. subtilis* is not a halophile but it can grow over a considerable range of osmotic conditions. It can contaminate food but rarely causes food poisoning and is not considered as a human pathogen. Historically, *B. subtilis* was a term given to all aerobic endospore-forming bacilli. Later, *B. subtilis* and two closely related species, *B. licheniformis*, and *B. pumilus*, were grouped taxonomically into what was known as the subtilis-group. However, recently methods have been developed that allow *B. subtilis* to be distinguished from these other species. The *B. subtilis* complete genome was sequenced in 1997 (Kunst et al., 1997). It contains 4,214,810 base pairs which comprise 4,100 protein-coding genes. The identification of five signal peptidase genes, as well as several genes for components of the secretion apparatus, is important given the capacity of *Bacillus* strains to secrete large amounts of industrially important enzymes.

2. Significance of water for living cells

In addition to the availability of nutrients, varying temperatures and pH, another frequently changing factor from the living cells environment, is the salt concentration, which is not only

prevalent in saline habitats, but also common in soil, in which rainfall and evaporation cause drastic changes in the surrounding osmolarity.

Each bacterial cell is enclosed by a semipermeable cytoplasmic membrane that restricts the free movement of most ions and metabolites, but not of water. In general, the total concentration of osmotically active solutes within a cell is higher than that in the environment. This causes the osmosis, i.e. the diffusion of water down its chemical potential into the cell which tends to reduce the difference in concentrations. The pressure that is required to maintain this equilibrium is termed osmotic pressure. Since it has meaning only in the context of one solution separated from another by a semipermeable membrane, a more meaningful term describing the osmotic property of solution is the osmotic potential (Π). It is defined as follows:

$$\mathbf{\Pi = - (RT / V_w) \ln a_w}$$

where **R** is the universal gas constant, **T** is the absolute temperature (Kelvin), $\mathbf{V_w}$ is the partial molar volume of water and a_w is the activity of water.

Osmotic pressure can also be expressed in terms of osmolality – units of osmols per kilogram of solvent

$$\text{osmolality} = \mathbf{\Pi / RT}$$

The term osmolarity is frequently used approximation of osmolality and is defined as the sum of the concentration of osmotically active particles in solution (**Wood, 1999**)

$$\text{osmolarity} = \mathbf{\Sigma_i \, c_i \approx \Pi / RT}$$

In bacterial and plant cells, the semipermeable cytoplasmic membrane is surrounded by the cell wall. As the water diffuses into the cell, a hydrostatic pressure is build up which lead to a pressure of the cell content against the cell wall, i.e. turgor pressure. In practice, the cells are not surrounded by pure water but by nutrient solutions of various osmolarities. Hence, the turgor pressure of the cells is the hydrostatic pressure difference which balances the osmotic pressure difference between cell interior and exterior. Turgor is maintained throughout the growth cycle as the cell elongates, and is considered to be necessary for enlargement of the cell envelope and, thus, for growth and division (Chater et al., 1999). The turgor pressure of bacterial cells can be calculated by different methods (Csonka, 1989) and the measurements indicate that it is considerably greater in gram-positive than in gram-negative bacteria, with values of 15 to 20 atm for the gram-positive and 0.8 to 5 atm for gram-negative organisms (Csonka and Epstein, 1996; Ingraham and Marr, 1996). In *B. subtilis*, turgor has been estimated at 19 atm (Whatmore and Reed, 1990).

Water fluxes across the cytoplasmic membrane are accomplished by simple diffusion across the lipid bilayer or through the so-called aquaporins. These are membrane water channels which are widely distributed in all kingdoms of life, including bacteria, plants, and mammals. The mechanisms underlying water transport are investigated (Tajkhorshid, 2002) and crystallographic structures of several aquaporins have been solved (Wang, 2005). The *E. coli* aquaporin (AqpZ) serves as a model for bacterial water channels (Calamita et al., 1995). However, in the *B. subtilis* genome no AqpZ-related proteins are encoded (Calamita, 2000) which implys that it does not contain aquaporins or it possesses water channel of a yet unknown type.

3. Osmosensing and osmoregulation in bacteria with focus on *B. subtilis*

Fluctuations of the external salinity and osmolarity are the most common type of environmental stresses that affect the growth and survival of all kind of cells, both of prokaryotic and eukaryotic origin (Galinski and Trüper, 1994; Csonka and Epstein, 1996; Bremer and Krämer 2000; Roeßler and Müller, 2001; Bremer, 2002; Morbach and Krämer, 2002; Holtmann et al., 2004; Wood, 2006; Tuteja, 2007). Microorganisms can colonize different ecological niches which comprise a wide variety of osmotic conditions. The cells try to keep their volume and turgor constant when a hypo- or hyperosmotic stress is present in the surrounding area.

3. 1. Osmoregulation in response to hypoosmotic conditions

Rain, flooding and washout into freshwater sources expose *B. subtilis* and other soil bacteria to drastic osmotic dowshocks which is life threatening. The water enters into the cell and increases turgor pressure which could lead to bursting of the cell. To avoid this, microorganisms activate the so-called mechanosensitive channels (MS channels). These are safety valves which are responsible for the rapid release of the water-attracting osmolytes and ions from the cell (Blount and Moe, 1999; Booth and Louis, 1999). Such channels have been discovered in different organisms including archaea, bacteria, fungi, plants and mammalian cells (Morris, 1990; Sackin, 1995; Sukharev et al., 1997; Kloda and Martinac, 2001). In *E. coli*, three different MS channels were identified with patch-clamp analysis (Morbach and Krämer, 2002). These are MscL, MscS and MscM - mechanosensitive channel of large, small or mini conductance, respectively. The genes coding for MscL and MscS were identified,

whereas the one for MscM is still unknown. The simultaneous disruption of both genes caused cell death after a severe osmotic downshock (Levina et al., 1999).

In *B. subtilis*, the presence of tension-gating pores was demonstrated with electrophysiological studies. The MscL protein is evolutionary well conserved (Moe et al., 1998) and a single copy of *mscL* gene is found in *B. subtilis*. Also three YggB homologues (the major component of MscS activity) that might function as MscS-type channel are also present in *B. subtilis* (Levina et al., 1999). However, the disruption of those three genes did not yielded a significant phenotype of the respective mutants in the hypoosmotic conditions. On the contrary combination of *mscL* deletion, together with one of the genes (*ykuT*) led to an extreme sensitivity of the *B. subtilis* strain (Holtmann et al., 2004). This indicated that the detected MS chanels of MscL and MscS-type play an important role in the adaptation to the low osmotic conditions.

3. 2. Osmoregulation in response to hyperosmotic conditions

When the bacteria are challenged by an increase in the surrounding osmolarity, the water efflux, the cell dehydrates and this could cause plasmolyse. In order to re-establish the turgor pressure and to prevent the loss of water, the living cells developed two strategies, which are referred to as "salt-in" and "salt-out" (Galinski and Trüper, 1994). The first strategy can be found in extremely halophilic *Halobacteria* (*Archaea*) and halophilic, anaerobic *Haloanaerobiales* (*Bacteria*) (Ventosa et al., 1998). These organisms can accumulate in their cytoplasm molar concentrations of K^+ and Cl^-, and actively expel the cytotoxic Na^+. Consequently, these extreme halophiles require high salt concentrations (mostly K^+) for their biochemical reactions. This "salt-in" strategy is effective only in habitats where high salt concentrations are permanently present, while in the environment with frequent fluctuation in the osmolarity the letter is not an advantage.

Much more flexibility is proposed by the "salt-out" strategy. Microorganisms, including *B. subtilis*, which live in habitats with moderate osmolarities or in environments with often changes in the surrounding conditions with respect to water activity, use this adaptation response. They cope with the high ionic conditions in their cytoplasm by uptake or synthesis of large amounts of specific organic osmolytes, the so-called compatible solutes (Brown, 1976). They are defined as small, highly soluble, organic molecules that do not interfere with the central metabolism and protein function, even if they are accumulated to high concentrations. This accumulation of compatible solutes upon osmotic stress is widely

distributed not only in Bacteria (Csonka and Epstein, 1996; Bremer and Krämer, 2000; Wood 2006) and Archaea (Roeßler and Müller, 2001; Roberts, 2004) but also in fungal, plant, animal and human cells (Dijksterhuis and de Vries, 2006; Yancey, 2005).The spectrum of compatible solutes used across the kingdoms comprises amino acids (proline and glutamate) and their derivatives (proline betaine and ectoine), small peptides (N-acetylglutaminylglutamine amide), methylamines and their sulfonium analogs (glycine betaine, carnitine and dimethylsulfoniopropionate), sulphate esters (choline-O-sulfate), polyols (glycerol and glucosylglycerol) and sugars (trehalose) (Bremer and Krämer, 1998; Da Costa et al., 1998). However, in the past year as more organisms have been studied, novel compatible solutes have been identified (Akashi et al., 2001; Alarico et al., 2007). Early work already suggested that the accumulation of compatible solutes is not only simple physical counterbalancing of decreasing water activity (Yancey et al., 1982; Le Rudulier et al., 1984). They also serve as stabilisers of proteins against the denaturing effects of high ionic strength. In parallel, this stabilizing effect confers increased tolerance also towards dessication, freezing and elevated temperatures. Trehalose, for example, was shown to bring about an increased desiccation tolerance to *E. coli* (Welsh and Herbert, 1999) and hydroxyectoine was involved in the thermoprotection of *Chromohalobacter salexigens* (Garcia-Estepa et al., 2006). The stabilizing effect of the compatible solutes is explained with the so-called preferential exclusion model (Bolen and Baskakov, 2001). In general, a protein is stabilized when it is kept in the native, folded state and the compatible solutes "force" the protein to stay in this correct folding. They are preferentially excluded from the immediate vicinity of proteins, implying an unfavourable interactions between the compatible solute and the protein surface, called the "osmophobic effect". As denatured proteins expose much more of the protein surface to the solvent compared with proteins in the folded state, the osmophobic effect "forces" proteins to fold correctly.

3.2.1. The initial stress response

When bacteria are subjected to a sudden osmotic upshift they usually respond with an adaptation reaction that is characterized by a rapid accumulation of K^+. Extensive studies have been performed with *E. coli*, which has four K^+ uptake systems (Epstein, 2003). Among them, the multicomponent Trk and Kdp systems play a role in the osmostress response. Also, in *Synechocystis* sp. Strain PCC 6803, the Ktr-like K^+ uptake system slr1509 (*ntpJ*) seems to be responsible for counteracting salt stress (Berry et al., 2003). Following an osmotic upshift,

an increase in the internal K^+ concentration has been detected in *Listeria monocytogenes* (Sleator, 2003) and *Corynebacterium glutamicum* (Morbach and Krämer, 2003), but the responsible K^+ uptake systems are not known so far. Recent studies with the halophilic bacterium *Halomonas elongata* revealed that al lower salinities, K^+ was the predominant cytoplasmic solute, whereas at higher salinities, ectoine became the dominant cytoplamic solute, while the K^+ content remained unchanged (Kraegeloh and Kunte, 2002). On the contrary, in *Saccharomyces cerevisiae* no ion uptake is reported (Hohmann, 2002).

It was found that after a sudden osmotic upshock *B. subtilis* also respond to by an initial rapid uptake of K^+ although not to a very high extent. The K^+ level rises from a basal value of 350 mM to approximately 650 mM within one hour (Whatmore et al., 1990). The inspectation of the *B. subtilis* genome revealed the presence of two major K^+ transporters: the high-affinity KtrAB system and the low-affinity KtrCD system (Holtmann et al., 2003). However, the expression of the *ktr* genes is not induced when the cells are subjected to high salinity. On the other hand the disruption of KtrAB or KtrCD led to major defects in K^+ uptake, and mutanrs defective in KtrAB transporter exhibited growth defects when imposed to a sudden osmotic upshock or continuously cultured in increased salinity (Holtmann et al., 2003). The influx of K^+ is important for the recovery of turgor and the resumption of growth after an osmotic challenge (Whatmore and Reed, 1990). These data indicated the importance of K^+ accumulation for the initial and the prolonged adaptation of *B. subtilis* to eleviated external osmolarity. Recently it was also demonstrated that K^+ uptake is also important for the ability of *B. subtilis* to colonize surfaces by spreading (Kinsinger et al., 2005).

Since the substantial accumulation of K^+ would lead to a charge imbalance in the cell, a respective counter-ion is needed to overcome this effect. Studies with the enteric gram-negative bacteria *E. coli* and *S. typhimurium* revealed that concomitantly with K^+ influx, within 1 min of the osmotic upshock, glutamate synthesis in increased to provide the counter-ion (McLaggan et al., 1994). Glutamate synthesis is dependent on the prior uptake of K^+, and glutamate is required to maintain the steady-state K^+ pool (Yan et al., 1996). In *B. subtilis* the nature of the counter-ion for K^+ is unclear so far because the level of the glutamate is only slightly increased after an osmotic upshock (Whatmore et al., 1990).

3.2.2. Osmoadaptation via biosynthesis of compatible solutes

Despite the importance of K^+ accumulation in the initial step of adaptation, high intracellular concentrations of this ion interfere with many important cellular processes like protein

function and DNA-protein interactions. Thus, the initial phase of osmoadaptation by means of K^+ influx is followed by accumulation of compatible solutes, which allows the cell to discharge the large amounts of K^+ (da Costa et al., 1998; Kempf and Bremer, 1998; Bremer and Krämer, 2000; Roeßler and Müller, 2001). The accumulation of compatible solutes occurs through synthesis or uptake from the environment. The most important compatible solutes used by *Bacteria* are trehalose, glycine betaine, proline and ectoine (Bremer and Krämer, 2000).

The sugar trehalose can be accumulated via de novo synthesis by *E. coli*, *S. typhimurium* and *C. glutamicum* (Dinnbier et al., 1988; Kempf and Bremer, 1998; Wolf et al., 2003). The trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase encoded by the *otsAB* operon are responsible for its production. On the other hand, trehalose is accumulated not only under salt stress but also under carbon-starvation conditions, which revealed its role as a general stress protectant (Strom and Kaasen, 1993). However, it is not found as a compatible solute in *B. subtilis*.

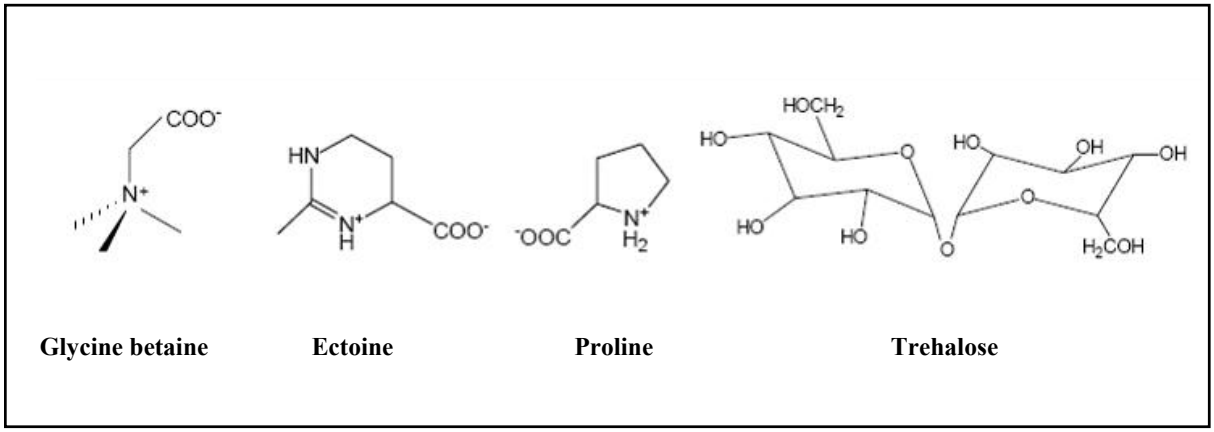


Fig. 1. Chemical structures of the commonly used compatible solutes in bacteria

Proline plays a crucial role in osmotolerance in many bacteria. It is also widely used in plant cells as a defence again high salinity and drought (Vendruscolo et al., 2007; Tuteja, 2007). In bacteria, synthesis of proline for anabolic purposes starts from glutamate and proceed in three enzymatic steps involving γ-glutamyl kinase (ProB), glutamate-semialdehyde-dehydrogenase (ProA), and pyrroline-5-carboxylate-reductase (ProC). The activity of ProB is frequently controlled by feedback inhibition trough proline. Increased concentrations of this compatible solute are found in *proB* mutants of *S. typhimurium* and *E. coli*, where single amino acid change prevents the feedback inhibition of the γ-glutamyl kinase (Csonka, 1988; Smith, 1985). In *B. subtilis*, proline production is strongly increased upon osmotic upshock with

0.4M NaCl (Whatmore et al., 1990) from a basal level of 16 mM to approximately 700 mM within 7 hours. The intracellular proline content is related to the external osmolarity of the medium in a linear fashion (Brill and Bremer, unpublished data). The anabolic pathway proceeds from glutamate trough ProB (γ-glutamyl kinase), ProA (γ-glutamyl phosphate reductase) and ProI/ProJ (pyrroline-5-carboxylate-reductase) (Belitsky et al., 2001). In parallel to that, a new proline biosynthetic pathway exists in *B. subtilis*, which is different from that used for anabolic purposes. The *proHJ* operon, encoding orthologues of the *proB* and *proI* genes, is needed in high-osmolarity stressed cells (Brill and Bremer, unpublished data). The *proHJ* mutant exhibits a severe growth defect under high osmotic conditions, which demonstrate the importance of de novo synthesized proline for osmoprotectance in *B. subtilis*.

Glycine betaine (N,N,N-trimethyl glycine) is one of the most widespread compatible solutes found in nature (Le Rudilier et al., 1984). The transfer of glycine betaine biosynthetic genes to plants resulted in transgenic plants with a significantly increased salt tolerance (Sakamoto and Murata, 2000). It has also protective function beyond the osmobalancing since it is accumulated in response to drought and cold (Welsh, 2000). Bacteria produce this trimethylated amino acid derivate in two different pathways. Some halophilic prototrophic eubacteria (Galinski and Trüper, 1994) and some extreme halophiles (Nyyssölä et al., 2000) can synthesize it de novo by a stepwise methylation of glycine. The second biosynthetic pathway is enzymatic oxidation of choline to glycine betaine, and it is used by both prokaryotic and eukaryotic cells, but the type of the involved enzymes can vary (Bremer and Krämer, 2000). *B. subtilis* uses the latter pathway since it can not synthesize glycine betaine de novo (Boch et al., 1994; Boch et al., 1996). For this purpose it utilizes a soluble, metal-containing, type III alcohol dehydrogenase (GbsB) to convert choline to glycine betaine aldehyde and a highly salt-tolerant glycine betaine aldehyde dehydrogenase (GbsA) to oxidize this toxic intermediate to the metabolically inert glycine betaine (Boch et al., 1997). The genes for these enzymes are encoded by the *gbsAB* operon. Since *B. subtilis* is unable to synthesize choline, it acquires this precursor from the environment through a highly substrate-specific ABC-transporter OpuB and a closely related broad substrate specific OpuC system (Kappes et al., 1999). The presence of exogenous choline stimulates the expression of the gene for its transport (*opuB*) and its enzymatic oxidation (*gbsAB*), whereas high osmolarity in the environment induces only the *opuB* gene (Nau-Wagner and Bremer, unpublished data). It was also found that the transcription from *opuB* and *gbsAB* is mediated by GbsR repressor, a novel type of choline sensing protein (Nau-Wagner and Bremer, unpublished data). It is

possible that GbsR-type proteins are widely spread in the gram-positive bacteria, since *gbsR*-related genes are present in *Staphylococcus xylosus* (Rosenstein et al., 1999) and *Staphylococcus aureus* (de Lancaster et al., 1999), in the vicinity of genes that are likely involved in glycine betaine synthesis. Although the broad-substrate specific OpuC system is involved in choline transport as well, it is not under the control GbsR.

In the genus *Bacillus*, the second most frequently synthesized compatible solute in response to high osmolarity is ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) (Kuhlmann and Bremer, 2002). As in the moderate halophile *Halomonas elongata* (Canovas et al., 1998), three enzymatic steps are required for the conversion of the precursor aspartate β-semialdehyde in ectoine (Ono et al., 1999). It was demonstrated that there is a linear relationship between the ectoine content and the external osmolarity in *Bacillus pasteurii* (Kuhlmann and Bremer, 2002). Nevertheless, *B. subtilis* can not produce this tetrahydropirimidine, but like many others bacteria, it can accumulate it for osmoprotective purposes with low affinity from the environment (Jebbar et al., 1997).

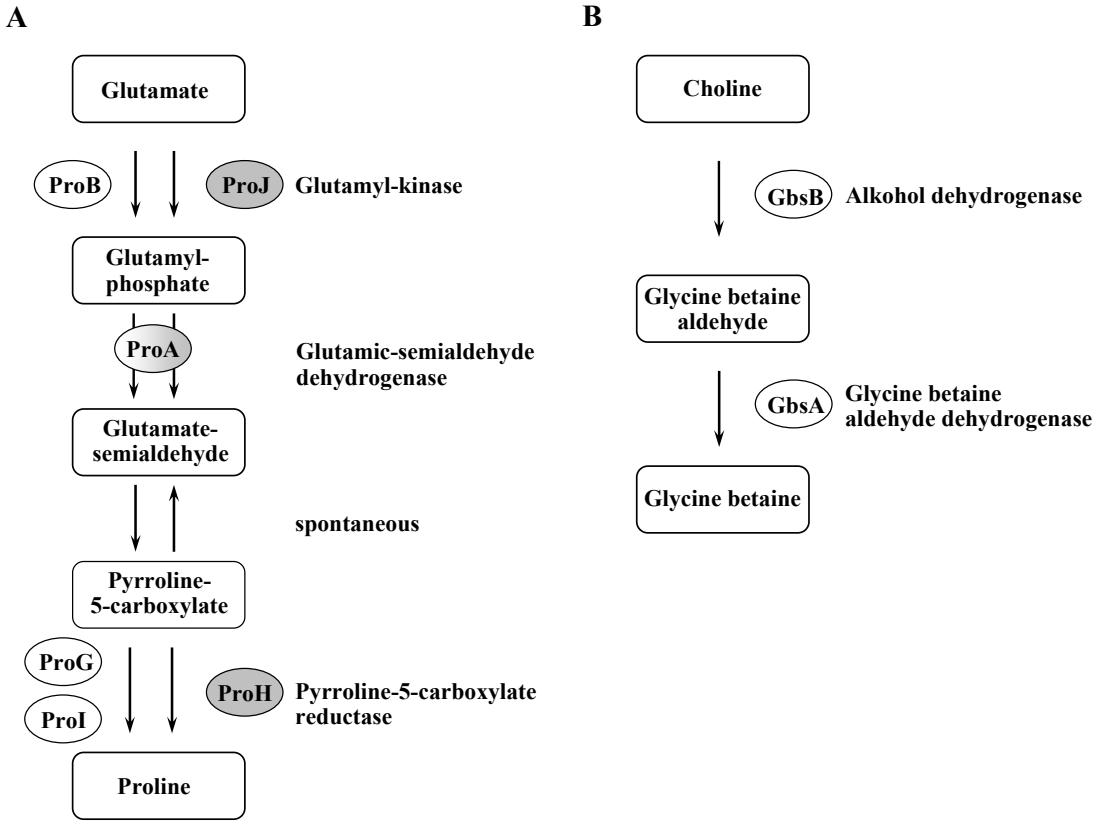


Fig. 2. Biosynthesis pathways for proline and glycine betaine in *B. subtilis*

A. Proline biosynthesis. Enzymes used for proline accumulation under osmotic stress conditions are on the right side (in grey) and those used for anabolic purposes are on the left side (no colour). The ProA is part from both pathways. **B.** Osmoregulatory synthesis for glycine betaine.

3.2.3. Transport of compatible solutes for osmoprotective purposes

In addition to accumulating compatible solutes by endogenous synthesis, bacteria are able to acquire them from exogenous sources. In contrast to hypoosmotic shock, only transport systems and not channels can be used for coping with hyperosmotic stress. Uptake has the advantage of being energetically cheaper than synthesis. In the ecosystems these compounds originate from osmotically downshocked microbial cells, decaying microbial, plant and animal cells, as well as from root exudates (Welsh, 2000). Genarally, the availability of osmoprotectants and their biosynthetic precursors in the environment is varying and usually very low, with concentrations in the nanomolar to micromolar range. This lead to the particular properties of most uptake systems for compatible solutes: (i) high affinity for their substrates, (ii) the capacity for high internal accumulation of the transported solute against its concentration gradient and (iii) high activity under conditions of increased osmolarity and ionic strength, where transporters for nutrients are generally inhibited (Roth et al., 1985). To take advantage of the diverse osmoprotectants in the surrounding habitat, microorganisms harbour multiple osmoregulatory transporters with overlapping substrate specificities. Different classes of transport systems were identified to function as compatible solute transporters. Namely, Na^+ or H^+ dependent secondary transporters – for example BetP and EctP from *C. glutamicum* (Morbach and Krämer, 2005; Weinand et al., 2007) or ProP from *E. coli* (Morbach and Krämer, 2002; Wood et al., 2005), or ABC transporters – for example ProU from *E. coli* (Lucht and Bremer, 1994) or OpuA from *L. lactis* (Bouvier et al., 2000).

In *B. subtilis* five transport systems (Opu – osmoprotectant uptake) for osmoprotectants have been found: two of them are secondary transporters (OpuD and OpuE), and three are members of the ABC (ATP binding cassette) superfamily (OpuA, OpuB and OpuC) (Kempf and Bremer, 1998) (Fig. 1). Each of these transporters exhibits a high affinity for its various substrates with K_m values in the low micromolar range (Kempf and Bremer, 1995; Kappes et al., 1996, 1999; von Blohn et al., 1997; Nau-Wagner et al., 1999; Horn et al., 2005). The high-affinity proline transporter OpuE is a member of the sodium-solute symporter family (SSF). The glycine betaine transporter OpuD is a member of the BCCT (betaine/choline/carnitine/transporters) family of secondary uptake systems. The OpuD transport activity is upregulated under high osmotic conditions (Kappes et al., 1996), whereas activity of the OpuE is not influenced form the medium osmolarity (von Blohn et al., 1997). The structural genes for OpuB and OpuC (*opuB* and *opuC*) are located closely on the chromosome and the high amino acid identities between them strongly suggest that both

transporters have evolved from a gene duplication event (Kappes et al., 1999). Despite of this identity, they exhibit substantial difference with respect to their substrate specificity. Namely, OpuB is highly specific for the uptake of choline, while OpuC can transport a wide range of compatible solutes, most of which are structurally related to glycine betaine.

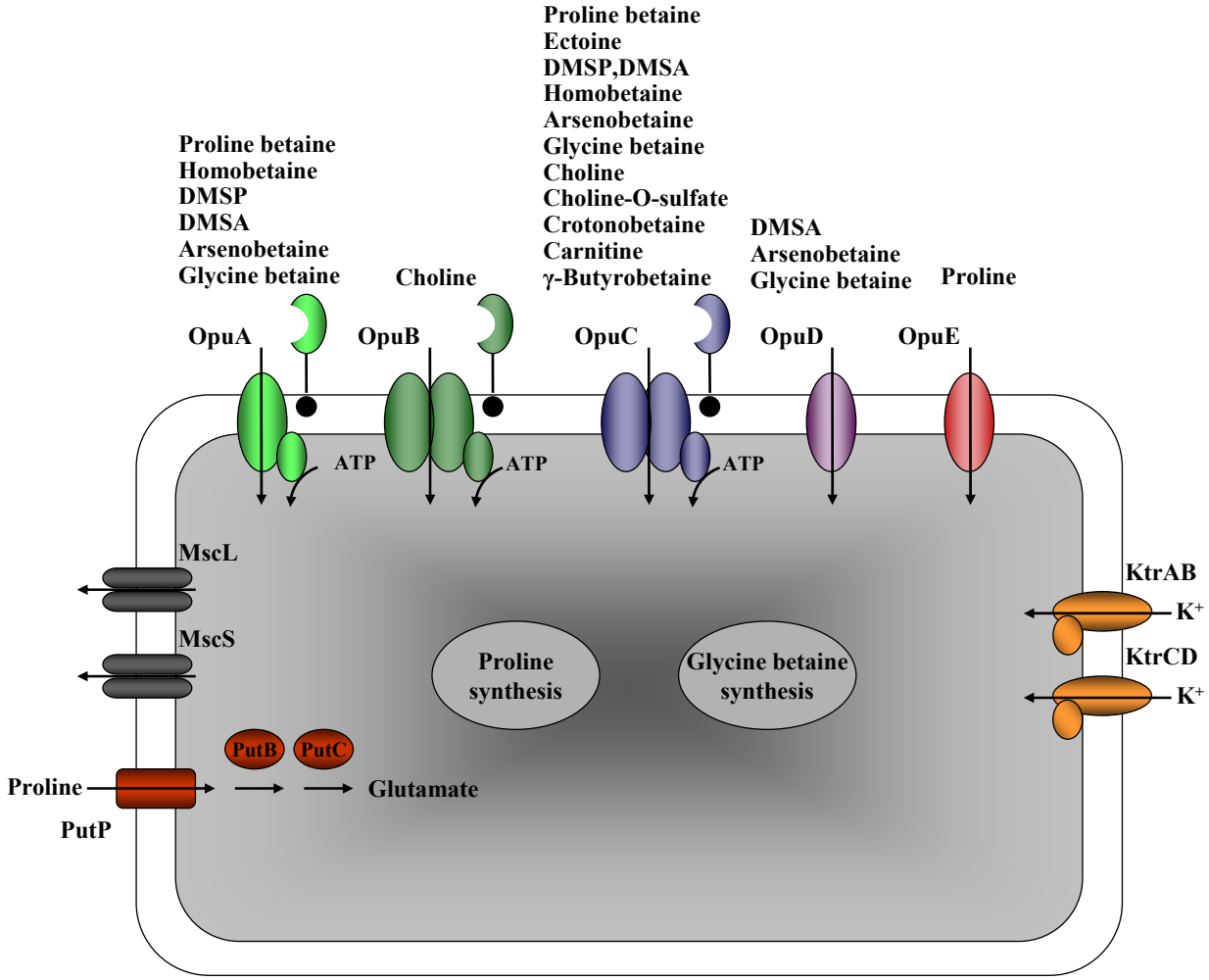


Fig. 3. Transport systems involved in the accumulation and expulsion of compatible solutes under high and low osmotic conditions in *B. subtilis* (Bremer, 2002)

With the exception of *opuB* expression, which is induced by choline and is under the negative control of the GbsR repressor (Nau-Wagner and Bremer, unpublished results), the expression of the *opuA*, *opuC*, *opuD* and *opuE* is not enhanced when the respective substrate for the transporter is present in the growth medium. However, transcription of the structural genes of the Opu transporters is induced in high osmotic environment and this induction is related linearly to the degree of the osmotic stress. Altogether twelve compounds are currently known

to function as osmoprotectants and compatible solutes for *B. subtilis*, and with the exception of ectoine, each of these compound is structurally related to either proline or glycine betaine. Nevertheless, the mechanism through which *B. subtilis* senses osmotic changes in the environment and transmit the information to the transcription apparatus still remains elusive.

3.3. General stress response

B. subtilis is mostly found in a nongrowing state due to nutrient limitations and many stress factors. Under severe starvation it can overcome this unfavourable situation by forming highly dessication-resistant endospores (Sonenshein, 2000). Hence, the high osmolarity in the environment would lead to sporulation in *B. subtilis*. However, the high salt actually inhibits the spore formation perhaps by blocking sigma factors that are involved in the early stages of sporulation (Ruzal et al., 1998). In order to escape those stress conditions, *B. subtilis* activates a very large general stress regulon whose expression depends on the alternative sigma factor SigB (σ^B). σ^B is an alternative sigma factor which is usually activated early in the stationary growth phase but is not required for sporulation (Boylan et al., 1993). In addition, a variety of environmental stress factors and growth-limiting conditions including high salinity trigger the transient expression of the entire σ^B regulon (Bernhardt et al., 1997; Hecker and Völker, 1998; Price, 2002; Höper et al., 2006). Protein analysis has demonstrated that the proteins induced under these conditions can be devided into two groups: the salt-sprecific stress proteins and the general stress proteins, which can be further subdevided into SigB-dependent and SigB-independent classes (Bernhardt et al., 1997). *B. subtilis* mutants lacking σ^B are highly sensitive to sudden and growth-restricting upshock with NaCl (Völker et al., 1999) but this can be counteracted by the uptake of glycine betaine. The structural genes for both glycine betaine transporter OpuD and the proline uptake system OpuE (Spiegelhalter and Bremer, 1998) are members of the σ^B regulon. Both genes are transcribed from two independently controlled promoters that respond to osmotic stress. One of these promoters is recognized by the housekeeping sigma factor σ^A, and the second promoter is recognized by σ^B. The σ^B controlled promoters respond only transiently to a rapid osmotic upshift. In contrast, the level of transcription initiating from the σ^A controlled promoters is retained as long as the osmotic stimulus persist, suggesting that at least two different signal transduction pathways must operate in *B. subtilis* to connect the environmental osmotic changes with the transcription apparatus of the cell. Therefore, both the specific osmostress reactions (uptake of potassium and accumulation of compatible solutes) and the induction of the SigB-dependent general

stress response are likely to play important physiological roles for the effective adaptation of *B. subtilis* to changing osmolarity in its natural habitats.

4. Two-component regulatory systems

Unicellular organisms are routinely challenged by drastic changes in their extracellular environments such as nutrient starvation, temperature and osmolarity shifts and etc. Organisms that have the genetic capability to respond to altered conditions do so when they are stimulated by specific signals. Recognition of specific signals and conversion of this information into specific transcriptional or behavioural responses is the essence of signal transduction. The molecular mechanisms underlying many of these phenomena involve so-called two-component regulatory systems, in which one component (histidine kinase) of the system senses an environmental or cytoplasmic stimulus and transfers the stimulus to the other component (response regulator) which in turn can elicit a specific response comprising in most cases transcriptional activation of certain target genes. Such signalling systems that mediate such responses are of interest, not only in representing a fundamental strategy for intracellular information processing, but also as potential targets for antimicrobial drug development. The term "two-component" was introduced to describe a new class of regulatory systems found in bacteria (Nixon et al., 1986). The first model for two-component signal transduction was proposed from Ninfa et al. (Ninfa et al., 1988). To date, researchers have found hundreds of such systems in all major branches of bacteria and archaea as well as in eukaryotes, and they are not restricted to unicellular organisms. Though far less numerous than in bacteria, histidine kinases and response regulators are present also in fungi and plants (Chang and Stewart, 1998; Perraud et al., 1999). The only major group lacking these systems is the animal kingdom. The number of two-component proteins within different organisms can vary substantially – from 0 in *Mycoplasma genitalium* (Fraser et al., 1995) to more that 80 in *Synechocystis* (Mizuno et al., 1996). In contrast, only a limited number have been found in eukaryotes – in the genome of *Saccharomyces cerevisiae* there is only one phosphorelay system involved in osmoregulation (Posas et al., 1996); the pathogenic fungus *Candida albicans* contains at least two HKs involved in osmoregulation and hyphal development (Nagahashi et al., 1998; Alex et al., 1998). Two-component proteins have been found also in plants, such as *Arabidopsis thaliana* (Hua et al., 1995) and tomato (Yen et al., 1995). Physiological processes that involve sensing through histidine kinases (HK) include cell differentiation and cell cycle regulation, chemotaxis and motility, nitrogen and phosphate

homeostasis, production of and resistance to antibiotics, quorum sensing and genetic competence, setting of circadian rhythms and fruit ripening, regulation of turgor and sugar transport, virulence and pathogenicity and etc (Grebe and Stock, 1999).

4.1. System architecture

The majority of two-component systems have a very simple design. The prototypical system consists of a histidine proteine kinase (HK) and a response regulator protein (RR). Extracellular stimuli are sensed by and serve to modulate the activities of the HK. HKs catalyze the ATP dependent phosphorylation of a conserved His residue via a trans-autophosphorylation mechanism whereby one HK monomer phosphorylates a second monomer within the HK dimer. The phosphoryl group from the HK is then transferred to an Asp residue in the cognate RR. His phosphorylation occurs on nitrogen, producing a phosphoramidate bond. This high energy, acid labile N-P bond has a large negative free energy of hydrolysis. The equilibrium of the HK autophosphorylation reaction favours the unphosphorylated protein. At typical intracellular ATP/ADP ratios, only a small percentage of the HK is phosphorylated. Phosphorylation of the Asp residue within the RR produces a high-energy acyl phosphate which is thought to be used for driving conformational changes in the protein. Phosphotransfer to the RR results in its activation that elicits the specific response (Stock et al., 2000). The lifetime of phospho-Asp within RRs varies significantly. Typical half-lives range from seconds to hours (Makino et al., 1989; Wright et al., 1993). Many RRs have autophosphatase activities that decrease the lifetime of the phosphoprotein (Hess et al., 1988). In a few cases, The RR stabilizes the phosphor-Asp, increasing the half-life significantly beyond that of a typical acyl phosphate (Janiak-Spens et al., 1999). Finally, the phosphoryl group is transferred from the phosphor-Asp residue to water in a hydrolysis reaction. All three reactions require divalent metal ions, with Mg^{2+} presumably being the relevant cation in vivo (Stock et al., 2000).

More complex scheme of the two-component systems are the phosphorelays (Perraud et al., 1999). They involve multiple His-containing and Asp-containing domains and a four step His-Asp-His-Asp phosphotransfer. Phosphotransfer pathways are the most common architecture in prokaryotes, while phosphorelays, which provide a greater number of steps for regulation, are predominant in eukaryotes. The first system with a four member relay was discovered in *Bacillus subtilis*, where it governs the events leading to sporulation (Burbulys et al., 1991). There are number of proteins in both pro- and eukaryotes that are unrelated to the histidine

protein kinase superfamily but are nevertheless phosphorylated on histidines (Grebe and Stock, 1999).

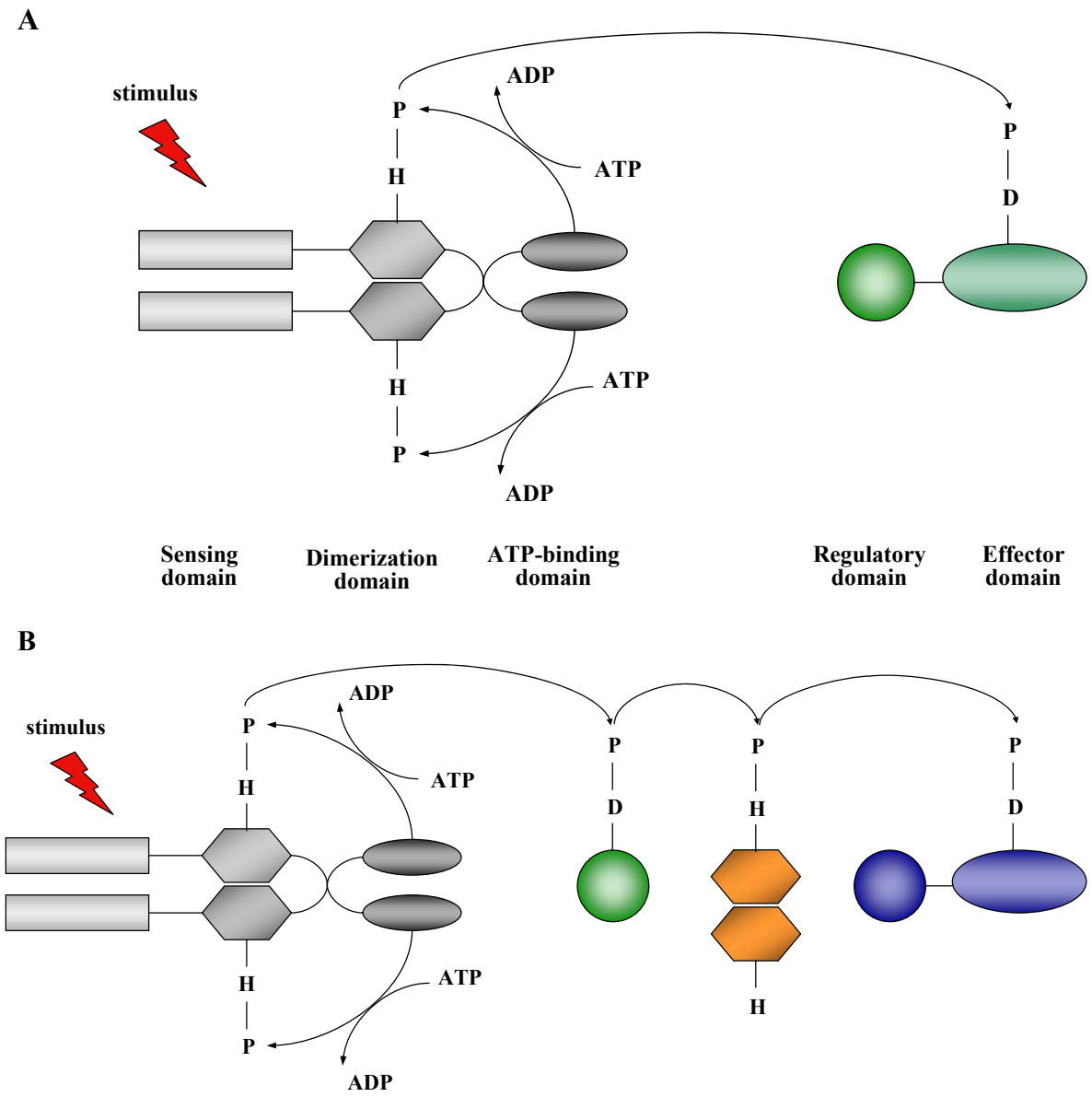


Fig. 4. Model of two-component mediated signal transduction

A. Typical two-component phosphotransfer system. The sensor kinase is autophosphorylated upon stimulus perceiption and consequently it transfers the phosphate to its cognate response regulator. **B.** Multicomponent phosphorelay. The signal transduction is accomplished by the interaction of several proteins whereby more than one His-Asp phosphotransfer takes place.

4.2. Structure of histidine kinases

HKs can be devided into two classes: orthodox and hybrid kinases. Most orthodox HKs function as periplasmic membrane receptors. The EnvZ protein from *E. coli* represents the most common membrane topology and contains two transmembrane regions (Yoshida et al.,

2007), whereas other HKs have multiple transmembrane segments (Lois et al., 1993; Island et al., 1992). Some TCS utilize soluble HKs regulated by intracellular stimuli or protein-protein interactions with other cytosolic components. Such examples are the chemotaxis kinase CheA (Stock et al., 1988) and the nitrogen regulatory kinase NtrB (MacFarlane and Merrick, 1985). The more elaborate hybrid kinases are found in some prokaryotic and most all eukaryotic systems. These proteins contain multiple phosphodonor and phosphoacceptor sites. Instead of promoting single phosphoryl transfer, hybrid kinases use multistep phosphorelay schemes (Hoch, 2000; West and Stock et al., 2001; Wolanin et al., 2002). In the phosphorelays, an intermediate histidine phosphotransfer protein (HPt) is involved either as a soluble protein or as an attached carboxy-terminal domain of the hybrid HK. Typical bacterial representatives are ArcB (Ishige et al., 1994) and CheA proteins (Mourey et al., 2001). The HKs typically contain two functionally and structurally distinct parts, a varialble N-terminal sensor region and a conserved C-terminal kinase core domain. The characteristic feature of the HK family is the kinase core. It is composed of a dimerization domain and an ATP-binding phosphotransfer or catalytic domain (Stock, 1999). The kinase core is about 350 amino acids in length and is responsible for binding ATP and directing kinase transphosphorylation. There are five conserved amino acid motifs present in both prokaryotic and eukaryotic HKs (Stock et al., 1989; Parkinson and Kofoid, 1992). The conserved His is the central feature in the H box, whereas N, G1, F and G2 boxes define the nucleotide binding cleft. In most HKs, the H box is part of the dimerization domain. However, for some proteins the conserved His is located at the far N-terminus of the protein in a separate HPt domain. The N, G1, F and G2 bosex are usually closely situated, but the spacing between them can vary. In archetypal HKs, the conserved His is located within the dimerization domain, adjacent to the nucleotide binding domain. The dimerization domain is made up of two antiparallel helices which form a four-helix bundle upon dimerization (Tomomori et al., 1999). His-containing domains function as intermediates in the phosphotransfer pathway, accepting phosphoryl groups from upstream donors (either a HK bound ATP or a phosphor-Asp of a RR domain) and transferring them to downstream RR domains. Therefore, residue surrounding the conserved His are expected to be involved in phosphotransfer catalysis as well as protein-protein recognition. Indeed, patterns of conserved sequences surrounding the His have been noted, but discrete functions remain to be ascribed (Fabret et al., 1999; Xu and West, 1999). According to the sequence similarities in their kinase domain, the histidine kinases are devided into 11 subfamilies (Grebe and Stock, 1999).

Environmental stimuli are detected either directly or indirectly by the N-terminal sensing domain of the HK. These diverse sensing domains share little primary sequence similarities, supporting the idea that they have been designed for specific ligand/stimulus interactions. In numerous cases, the specific stimuli and mechanism of sensing remain undefined. Cytosolic sensing modules, such as PAS and GAF domains, have been identified in numerous HKs. PAS domains named for the PER, ARNT and SIM proteins, are an evolutionary related family of versatile signalling modules whose functions are dependent upon an associated cofactor (Taylor and Zhulin, 1999; Pandini and Bonati, 2005). In bacteria and archaea, these domains are almost exclusively found within HKs, whereas in eukaryotes they are involved in a diverse set of regulatory functions. Some kinases lack the sensing domain.

4.3. Structure of response regulators

Most RRs have two-domain architecture with an N-terminal regulatory domain linked to a C-terminal effector domain. HK mediated phosphorylation of the conserved Asp residue in the regulatory domain creates an active RR, capable of eliciting an intracellular response. Members of the RR family mediate a diverse set of output responses dedicated by their effector domains The majority of RRs are transcription factors that contain helix-turn-helix DNA-binding effector domains that can be devided into three major subfamilies based on the homology of their DNA-biniding domains: the OmpR/PhoB, the NarL/FixJ and the NtrC (West and Stock, 2001). Nevertheless, there are a few exceptions where the C-terminal domains function as enzymes, such as the chemotaxis methylesterase CheB (Simms et al., 1985). Although typically found within multidomain RRs, regulatory domains are not always attached to effector domains. Some exist as separate proteins at the ends of pathways where they mediate intermolecular regulation of output responses (Welch et al., 1993), while others are used in phosphorelay pathways as intermediates or as domains of hybrid HKs (Burbulys et al., 1991). In *B. subtilis* there are five response regulators (Spo0F, CheY, YneI, CheV and CheB) do not possess a C-terminal DNA-binding domain (Kobayashi et al., 2001). These appear to play roles in protein-protein interactions rather than in direct regulation of genetic expression.

How effector domain function of the RR is modulated by phosphorylation of the regulatory domain has been a central question. One hypothesis has emerged according to which, RRs exist in equilibrium between two predominant conformations, corresponding to the inactive and active states. Phosphorylation shifts the equilibrium towards the active conformer.

Activation alters a large molecular surface of the regulatory domain, subset of which dictates specific protein-protein interactions that mediate the output response. Structural studies of constitutively activated mutant RR proteins have given indications of conformational change associated with activated regulatory domains (Zhu et al., 1997; Nohaile et al., 1997). From here rises the next question of how these structural perturbations regulate the output response of the RR. Given the number and diversity of RRs, there appear to be many strategies for regulation, even for proteins with common functions, such as the large family of RRs that function as transcription factors. In some cases, phosphorylation promotes dimerization that is required for DNA binding, while in other cases, phosphorylation enhances DNA binding in the absence of dimerization. Yet other RRs bind to DNA in the absence of phosphorylation, but require phosphorylation for productive interaction with transcriptional machinery (Robinson et al., 2000). Hence, regulation in different RRs involves fundamentally distinct molecular mechanisms.

His-Asp phosphotransfer systems account for the majority of signalling pathways in eubacteria but are quite rare in eukaryotes, in which kinase cascades involving Ser/Thr and Tyr phosphorylation predominates. Nevertheless, His-Asp systems have been found in several eukaryotic organisms (Loomis et al., 1998) and Ser/Thr and Tyr kinases and phosphatases have been identified in bacteria, respectively (Zhang, 1996). In most two-component systems, there is a one-to-one relationship between HK and RR. In addition to promoting forward phosphoryl transfer, some HKs also act as phosphatases. Trough these opposing actions, the HK regulates the phosphorylation level of the downstream RR, controlling the flow of information through the signalling pathway.

There are still several central questions regarding the functioning of the HKs and RRs. In many systems, the stimuli sensed by the HKs are not well defined, and the molecular mechanism of signal transmission from the sensing domain to the kinase core has not been determined. In RRs, the detailed mechanisms that connect the phosphorylation-induced conformational changes in regulatory domains to activation of effector domains remain to be elucidated. There are also many questions regarding regulation within individual pathways.

5. The DegS-DegU two-component system from *Bacillus subtilis*

Soil bacteria such as *B. subtilis* are subject to drastic variations in environmental conditions such as temperature, humidity, osmolarity and nutrient source availability. At the onset of stationary phase, faced with a depletion of essential nutrients, *B. subtilis* can adopt several responses including synthesis of macromolecule-degrading enzymes, competence for genetic transformation, increased motility and chemotaxis, antibiotic production, and finally, sporulation. Each of these responses is controlled by at least one two-component regulatory system. In *B. subtilis*, 36 sensor kinases and 35 response regulators have been found, among which each of 30 kinase-regulator pairs resides in an operon on the genome (Fabret et al., 1999; Kunst et al, 1997). Among these two-component systems, only the DegS-DegU displayed a higher expression level in high- versus low-salt growth conditions (Steil et al., 2003). Interestingly, the system controls various processes that characterize the transition form the exponential to the stationary growth phase, including the induction of extracellular degradative enzymes, expression of late competence genes and down regulation of the sigma D regulon, which encompasses the genes involved in motility, chemotaxis and autolysin production.

The corresponding genes, *degS* and *degU*, were initially defined by different classes of mutations leading either do deficiency of degradative enzyme synthesis or to overproduction of those enzymes (Henner et al., 1988; Kunst et al., 1988). It was suggested that *degS* and *degU* genes form an operon encoding a two-component system since no obvious transcriptional terminator sequence is present between the two genes (Msadek et al., 1990).

The DegS and DegU proteins have been purified (Mukai et al., 1990; Dahl et al., 1991) and it was shown that the first component, a histidine proteine kinase (DegS), is autophosphorylated at a conserved histidine residue in an ATP-dependent reaction. In a second step, the phosphorylgroup is rapidly transferred to an aspartate residue in the conserved N-terminal domain of the second component, the response regulator (DegU) (Dahl et al., 1991; Tanaka et al., 1991). For the belonging of the system to the His-Asp two-component systems testified also the fact that the DegS-phosphate was stable in both neutral and alkaline media but was labile in acidic conditions, whereas the DegU-phosphate was labile in alkali and slightly sensitive to acid (Mukai et al., 1990). Sequence similarities with other two-component systems and mutational analysis of both genes suggested that the His-189 residue of the DegS and Asp-56 of the DegU as likely candidates for the respective phosphorylation sites of the two proteins. In parallel to phosphorylation of cognate response regulator, the DegS kinase

possesses also phosphatase activities, i.e. it is able to dephosphorylate DegU (Tanaka et al., 1991; Dahl et al., 1992). Such activities were also demonstrated for other sensor proteins like EnvZ and NtrB, which were able to remove the phosphate from their cognate response regulators OmpR and NtrC, respectively (Keener and Kustu, 1988; Aiba et al., 1989).

An interesting feature of the DegS-DegU system is the cytoplasmic localization of the histidine kinase since it does not contain any siginificant hydrophobic domains. With the exception of the spo0B histidine kinase which is part of the phosphorelay of the competence development, all other histidine kinases from *B. subtilis* appear to be membrane-bound proteins.

The products of the DegS-DegU two-component regulatory system control positively several degradative enzymes – intracellular protease and several secreted enzymes like levansucrase, alkaline and metalloproteases, α-amilase, xylanase, β-glucanase. These enzymes are synthesized during the exponential growth phase (levansucrase) or during the early stationary phase (proteases, α-amilase). From here comes also the name of the genes "deg", designated to reflect their role in the regulation of the degradative enzymes. Deletion or disruption of the *degU* gene abolished both degradative enzyme synthesis and genetic competence, indicating that this gene is essential for both cellular functions (Dahl et al., 1991). A strain from which the *degS* gene was deleted and in which the *degU* gene was expressed from the *degS-degU* operon promoter developed normal genetic competence, but the rate of degradative enzyme synthesis was reduced compared with that of the parental strain *B. subtilis* 168 (Msadek et al., 1991). It has been also shown that the DegU is a positive regulator of *comK* expression (Ogura and Tanaka, 1996; Hamoen et al., 2000) which is involved in competence development. In fact, the DegU protein has two active conformations: a phosphorylated form which is necessary for the degradative enzyme synthesis and a nonphosphorylated form required for expression of genetic competence (Dahl et al., 1992). This is in agreement with the observation that the *degU146* mutant is deficient for degradative enzyme production but retains the wild type level of competence. This mutation leads to the replacement of the aspartate residue at position 56 by asparagines in the DegU amino acid sequence, which is the actual phosphoacceptor site of the latter (Dahl et al., 1991). The DegU146 mutant protein was purified and tested for its phosphoaccepting activities which demonstrated that it can not be longer phosphorylated from the DegS kinase (Dahl et al., 1992). The in vivo equilibrium between the two forms of DegU is presumably regulated by DegS in response to an environmental signal that is not yet identified.

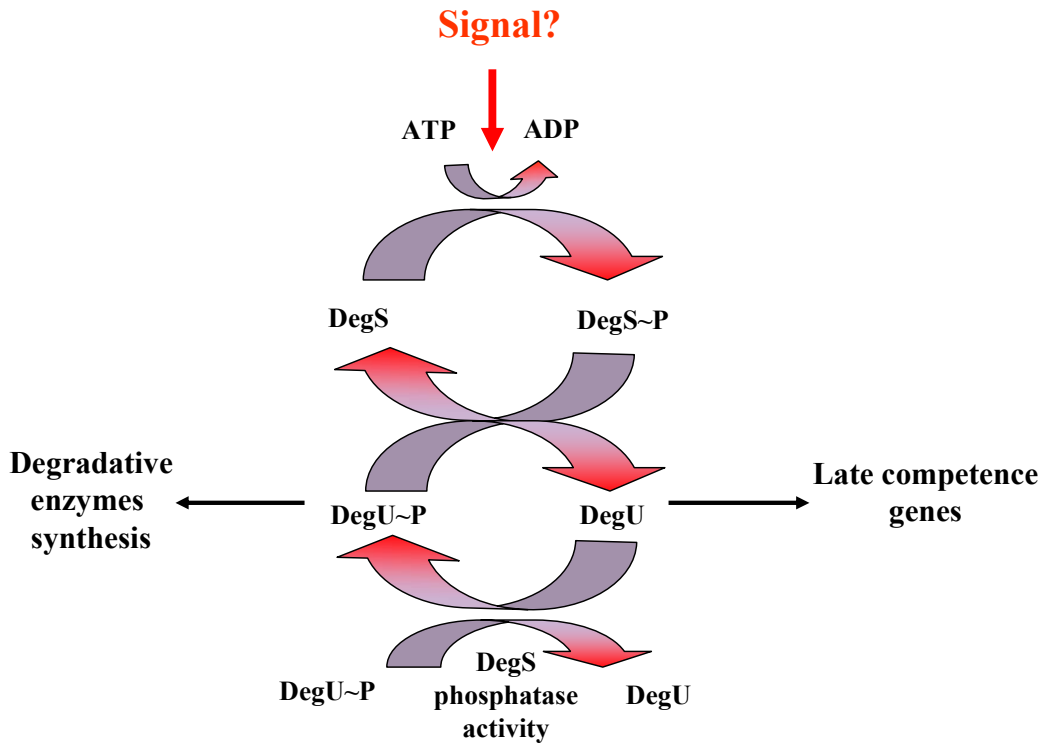


Fig. 5. Multi-level regulation by DegS-DegU two-component system

The DegU response regulator has two active conformations. The phosphorylated form is necessary for the degradative enzyme synthesis whereas the non-phosphorylated form is required for the development of genetic competence

It was shown that the *degU*(Hy) mutations lead to a pleiotropic phenotype including not only hyperproduction of degradative enzymes and deficiency in transformation, but also absence of flagella and capacity to sporulate in the presence of glucose. All the identified *degU*(Hy) mutations corresponded to modifications in the N-terminal receiver domain (Msadek et al., 1990). In contrast to the genetic competence and degradative enzyme synthesis, which require functional unphosphorylated or phosphorelated DegU protein respectively, sporulation and flagellar synthesis can occur in the absence of the *degS* and *degU* genes (Msadek et al., 1990). This indicates that these cellular functions do not require the presence of both genes, but they are influenced by mutations in the latter.

Recently, the DegS-DegU two-component system was implicated in another cellular function, i.e. production of γ-Poly-DL-glutamic acid (γ-PGA) and biofilm formation in the wild strain *B. subtilis* (Stanley and Lazazzera, 2005). The γ-PGA was indicated as an exopolymer that contributes to biofilm formation but is not absolutely necessary for that process. DNA microarray studies showed that transcription of *ywsC*, *ywtA* and *ywtB*, which are the γ-PGA biosynthetic genes (Urushibata et al., 2002), were activated by high levels of phosphorylated

DegU (Mäder et al., 2002). Nevertheless, the DegU is not the solely regulator that is part of the biofilm formation network and the involvement of other regulators like ComA, DegQ, Spo0A, AbrB led an open question of how these factors interact to each other and co-ordinate the biofilm formation.

One recent paper showed a novel role of phosphorylated DegU during the activation of swarming motility and regulation of complex colony architecture, an analogy of biofilm formation (Verhamme et al., 2007). Swarming motility is rapid and coordinated translocation of a bacterial population across solid or semi-solid surfaces, and is characteristic feature of wild strain *B. subtilis* only (Kearns and Losick, 2003). It was shown that very low levels of DegU~P, which can be generated in a *degS* mutant strain through an atypical pathway, are required to activate swarming motility. Because neither the *degS* nor the *degU* mutant strains exhibited a swarming motility defect, these findings indicated that *degU* is not required for flagellar biogenesis. In contrast, at high levels of DegU~P, swarming motility was inhibited. This is most likely a consequence of high levels of DegU~P inhibiting transcription of *fla-che* operon (Amati et al., 2004) that encodes the components for flagella biosynthesis, which is in turn required for swarming motility (Kearns and Losick, 2003). The same pattern was observed with respect to complex colony architecture, namely, the DegU~P is required for the complex colony architecture but in parallel high levels of the phosphorylated protein inhibited the same process. The authors proposed a model where DegU~P functions as a rheostat in regulating swarming motility, complex colony architecture and exoprotease production in a way where low levels of DegU~P are sufficient for activating the first two processes while high levels of the protein are required for activating the degradative enzyme production (Verhamme et al., 2007).

In addition to the other processes which are controlled by the DegS-DegU two-component regulatory system, the latter was implicated also in the salt stress activation. It was shown that the salt stress has a strong effect on the expression of *sacB* and *aprE* genes, encoding levansucrase and alkaline protease, respectively, and for which it was already known that are under the control of DegU protein (Kunst and Rapoport, 1995). However, it seemed that the expression from the *sacB* promoter was salt-specific and upregulated in the presence of 1M NaCl or 1M KCl, but not in the presence of high sugar concentrations. In parallel to that, the expression from *aprE* promoter was strongly decreased under these circumstances. The expression of the cell wall-associated gene *wapA* was also shown to be downregulated in the presence of phosphorylated DegU and high salt environmental conditions (Dartois et al., 1998). In addition, *degSU-lacZ* fusion assays demonstrated that the promoter region upstream

of *degS* gene is induced in hypertonic medium (Ruzal and Sanchez-Rivas, 1998). Finally, investigations of the transcriptional profiling of *B. subtilis* under high salt revealed that both *degS* and *degU* genes were up-regulated under those conditions (Steil et al., 2003). Microarray analysis of *B. subtilis* cells grown under hyperosmotic conditions revealed the induction of altogether 102 genes. Among them, the only representative of the two-component regulatory systems detected in *B. subtilis*, only DegS-DegU pair demonstrated higher induction when subjected to elevated salt concentrations (Steil et al., 2003). Separately, the DegS-DegU regulon has been characterized in transcriptome studies where the DegU-regulated genes were explored by the use of hyperactive DegU allele, *degU32Hy* (Mäder et al., 2002), or by overproduction of the regulator gene in the absence of the DegS kinase (Ogura et al., 2001). Steil and co-workers revealed that a big portion of the high-salinity induced genes in *B. subtilis* were also part of the DegU regulon as defined by Mäder at al, 2002, and Ogura et al., 2001. This implies for the substantial role of the DegS-DegU two-component system when the cell are subjected to high osmotic conditions.

Regardless of the fact that the processes which are regulated from DegS-DegU two-component regulatory system are well defined, one very important question still remains open. What is the molecular mechanism underlying the perception of a signal from the environment and its transmission to the DegS sensor kinase?

6. Aim of the work

The most common bacterial sensory systems which screen for changes in the environemental conditions and are responsible for the realization of the appropriate responses are the two-component regulatory systems (West and Stock, 2001). From the 36 histidine kinases and 35 response regulators detected in *B. subtilis* (Kobayashi et al, 2001), only the DegS-DegU two-component regulatory system was implicated in salt-induced response of the bacterium (Steil et al., 2003). Some of the genes, whose transcription was known to be influenced by the DegU response regulator, were also shown to be activated or repressed when subjected to high osmotic conditions. The aim of the current work was to characterize in more detail the involvement of the DegS-DegU two-component system in the osmotic regulation of *B. subtilis* at the transcriptional level and to explore the putative target genes which are activated upon osmotic induction through DegU response regulator.

When challenged with osmotic upshift, the organism must be able to recognize the altered conditions and to convert this information into specific transcriptional or behavioural responses. The induction of the degS and *degU* genes upon elevated osmolarities (Steil et al., 2003) suggested that the histidine kinase DegS may act as a sensor for osmostress-related stimuli. The osmotic activation of DegS could be triggered directly by a variety of parameters including a change in cell turgor, a change in the internal osmolarity, changes in the hydration state of DegS as a consequence of altered internal osmolarity, changes in the cytoplasmic ion concentration, or changes in the concentration of specific compounds interacting directly with the sensor kinase. The cytoplasmic localization of DegS prompted the question of how the information from the environment is transduced to the cytoplasm in order to stimulate its activation. Hence, in addition to the transcriptional regulation of DegS-DegU system, another objective of this work was to contribute to the understanding of the possible stimuli involved in the activation of the sensor kinase as a response to increased osmolarities.

III. Materials and methods

1. Chemicals and reagents

All chemicals were purchased from Amersham Biosciences (Freiburg, Germany), Applichem (Darmstadt, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Roche (Mannheim, Germany), Roth (Karlsruhe, Germany), Sigma-Aldrich (Deisenhofen, Germany) and were of the highest quality available. The restriction enzymes, DNA modifying enzymes and polymerases, used for the molecular biology experiments were from Amersham Biosciences (Freiburg, Germany), Eppendorf (Hamburg, Germany), Fermentas GmbH (St. Leon-Rot, Germany), New England Biolabs (Frankfurt am Main, Germany), Promega (Mannheim, Germany), Roche (Mannheim, Germany) or Stratagene (Amsterdam, NL).

2. Bacterial strains and plasmids

2.1. Bacterial strains

The *E.coli* and *B.subtilis* strains used in this study are summarized in Table 1 and 2, respectively.

Tab. 1: *B. subtilis* strains

Strain	Genotype	Reference/Source
JH642	*trpC2pheA1*	BGSC[a]
FSB1	JH642*(treA::neo)1*	Spiegelhalter, 1998
TRB0	JH642*(treA::neo)1amyE::('treA cat)*	Spiegelhalter, 1998
168	*trpC2*	BGSC
MD300	*degU32(Hy)aphA3-coupled*	Mäder et al., 2002
MD282	*degS degU::aphA3*	M.Dahl,University Konstanz, unpublished
GSB4	168*Δ(treA::neo)*	Lab strain
GNB37	JH642*Δ(treA::erm)*	Lab strain
JGB34	168*Δ(treA::erm)*	Lab strain
PB168	*trpC2*	Amati et al., 2004
PB5094	*trpC2 ΔdegSU*(Kan^r)	Amati et al., 2004
PB5213	*trpC2 degU32(Hy)*	Amati et al., 2004

PB5246	*trpC2 leuA8 degS200(Hy)*	Amati et al., 2004
THB1	168 Δ(*treA*::*neo*) *amyE::[Φ(degS'-treA)1 cat]*	This work
THB2	168 Δ(*treA*::*neo*) *amyE*::(*treA cat)*	This work
THB4	168 Δ(*treA*::*erm*)	This work
THB21	168 Δ(*treA*::*erm*) *amyE*::[Φ(*degS'-treA*)1 *cat*] *degS degU*::*aphA*3	This work
THB22	168 Δ(*treA*::*erm*)*amyE*::(*treA cat) degS degU*::*aphA*3	This work
THB31	168Δ(*treA*::*erm*)*amyE::[Φ(degS'-treA)1cat]degU*32(Hy)*aphA*3-coupled	This work
THB32	168 Δ(*treA*::*erm*) *amyE*::(*treA cat) degU*32(Hy)*aphA*3-coupled	This work
THB41	168 Δ(*treA*::*erm*) *amyE::[Φ(degS'-treA)1 cat]*	This work
THB42	168 Δ(*treA*::*erm*) *amyE*::(*treA cat)*	This work
THB210	168 Δ(*treA*::*erm*) *amyE*::[Φ(*yddT'-treA*)1 *cat*] *degS degU*::*aphA*3	This work
THB211	168Δ(*treA*::*erm*) *amyE*::[Φ(*yitMNOP'-treA*)1 *cat*] *degS degU*::*aphA*3	This work
THB212	168Δ(*treA*::*erm*) *amyE*::[Φ(*yitP'-treA*)1 *cat*] *degS degU*::*aphA*3	This work
THB213	168 Δ(*treA*::*erm*) *amyE*::[Φ(*yoaJ'-treA*)1 *cat*] *degS degU*::*aphA*3	This work
THB214	168 Δ(*treA*::*erm*) *amyE*::[Φ(*yomL'-treA*)1 *cat*] *degS degU*::*aphA*3	This work
THB215	168Δ(*treA*::*erm*) *amyE*::[Φ(*yqxIJ'-treA*)1 *cat*] *degS degU*::*aphA*3	This work
THB216	168Δ(*treA*::*erm*) *amyE*::[Φ(*degSU'-treA*)1 *cat*] *degS degU*::*aphA*3	This work
THB217	168Δ(*treA*::*erm*) *amyE*::[Φ(*degU'-treA*)1 *cat*] *degS degU*::*aphA*3	This work
THB253	JH642*(treA*::*neo*)1 *amyE*::[Φ(*degS'-treA)1 cat*]	This work
THB282	JH642 [*degS degU*::*aphA*3]	This work
THB300	JH642 [*degU*32(Hy) *aph*A3-coupled]	This work
THB310	168Δ(*treA*::*erm*)*amyE::[Φ(yddT'-treA)1cat]degU*32(Hy)*aphA*3-coupled	This work
THB311	168Δ(*treA*::*erm*)*amyE::[Φ(yitMNOP'-treA)1cat]degU*32(Hy)*aphA*3-coupled	This work
THB312	168Δ(*treA*::*erm*)*amyE::[Φ(yitP'-treA)1cat]degU*32(Hy)*aphA*3- coupled	This work
THB313	168Δ(*treA*::*erm*)*amyE::[Φ(yoaJ'-treA)1cat]degU*32(Hy)*aphA*3-coupled	This work
THB314	168Δ(*treA*::*erm*) *amyE::[Φ(yomL'-treA)1 cat] degU*32(Hy)*aphA*3-coupled	This work
THB315	168Δ(*treA*::*erm*) *amyE::[Φ(yqxIJ'-treA)1 cat] degU*32(Hy)*aphA*3-coupled	This work
THB316	168Δ(*treA*::*erm*) *amyE::[Φ(degSU'-treA)1 cat] degU*32(Hy)*aphA*3-coupled	This work
THB317	168Δ(*treA*::*erm*) *amyE::[Φ(degU'-treA)1 cat] degU*32(Hy)*aphA*3-coupled	This work

THB410	168 Δ(*treA*::*erm*) *amyE*::*[Φ(yddT'-treA)1 cat]*	This work
THB411	168 Δ(*treA*::*erm*) *amyE*::*[Φ(yitMNOP'-treA)1 cat]*	This work
THB412	168 Δ(*treA*::*erm*) *amyE*::*[Φ(yitP'-treA)1 cat]*	This work
THB413	168 Δ(*treA*::*erm*) *amyE*::*[Φ(yoaJ'-treA)1 cat]*	This work
THB414	168 Δ(*treA*::*erm*) *amyE*::*[Φ(yomL'-treA)1 cat]*	This work
THB415	168 Δ(*treA*::*erm*) *amyE*::*[Φ(yqxIJ'-treA)1 cat]*	This work
THB416	168 Δ(*treA*::*erm*) *amyE*::*[Φ(degSU'-treA)1 cat]*	This work
THB417	168 Δ(*treA*::*erm*) *amyE*::*[Φ(degU'-treA)1 cat]*	This work

[a] Bacillus Genetic Stock Center, Columbus, Ohio, USA

Tab. 2: *E. coli* strains

Strain	**Genotype**	**Reference/Sourse**
DH5α	*F⁻ λ⁻ E44Δ(argF-lac) U169φ80dlacΔ(lacZ)M15 hsdR17 recA1 endA1 gyr96 thi-1 relA1*	Hanahan, 1983
BL21	*F⁻ gal met r⁻m⁻ hsdS(λD3)*	Stratagene, Amsterdam

2. 2. Plasmids

In table 3 the plasmids used in this study are listed.

Tab. 3: Plasmids

Plasmid	**Description**	**Reference/Source**
pJMB1	Low-copy plasmid, carries a promoterless *treA* gene inserted into *amyE*, Amp^r, Cml^r	Jebbar and Bremer, 1997/unpublished
pRB373	*E. coli – B. subtilis* shuttle vector, Amp^r, Kan^r	Brückner, 1992
pASK-IBA3	Amp^r, cloning and expression vector, providing *tetA* promoter and C-terminal *Strep*-tag II	IBA, Göttingen
pOFXtac-SL2	Cml^r, *E.coli* vector, expression of *groES* and *groEL* under control of the pTac promoter	Castanie et al., 1997
pTH1	A derivative of pJMB1, carries *amyE*::[Φ(*degS*'-*treA)1 cat*]	This work
pTH2	A derivative of pRB373, degS', Amp^r, Kan^r	This work
pTH3	pASK-IBA3 derivative containing *degS* gene from *B. subtilis* in the *Bsa*I restriction site	This work
pTH4	pASK-IBA3 derivative containing *degU* gene from *B. subtilis* in the *Bsa*I restriction site	This work
pTH10	pJMB1 derivative, carries *amyE::[Φ(yddT'-treA)1 cat]*	This work
pTH11	pJMB1 derivative, carries *amyE::[Φ(yitMNOP'-treA)1 cat]*	This work

pTH12	pJMB1 derivative, carries *amyE::[Φ(yitP'-treA)1 cat]*	This work
pTH13	pJMB1 derivative, carries *amyE::[Φ(yoaJ'-treA)1 cat]*	This work
pTH14	pJMB1 derivative, carries *amyE::[Φ(yomL'-treA)1 cat]*	This work
pTH15	pJMB1 derivative, carries *amyE::[Φ(yqxIJ'-treA)1 cat]*	This work
pTH16	pJMB1 derivative, carries *amyE::[Φ(degSU'-treA)1 cat]*	This work
pTH17	pJMB1 derivative, carries *amyE::[Φ(degU'-treA)1 cat]*	This work

3. Oligonucleotides

All primers used in this study were synthesized from MWG Biotech (Ebersberg) or biomers (Ulm).

Tab. 4: Primers used for RNA-probes generation

Name	**Sequence (5'→3')**	**Description**
degS-NB-fwd	accgttgacgggagcaaggacg	Upstream region of *degS*
degS-NB-rev	taatacgactcactatagggaggcgctcttcttcctgcgcctc	Downstream region of *degS*, fused with T7 promoter
degU-NB-fwd	cctacctttgaagtggtagccg	Upstream region of *degU*
degU-NB-rev	taatacgactcactatagggaggccgtctgcaagcatctgcagc	Downstream region of *degU*, fused with T7 promoter
mpr-NB-fwd	ccgccccttatgagggaaccgg	Upstream region of *mpr*
mpr-NB-rev	taatacgactcactatagggaggcgaggaaaggcccacgggac	Downstream region of *mpr*, fused with T7 promoter
yddT-NB-fwd	cgcttctgcaaaggaggactc	Upstream region of *yddT*
yddT-NB-rev	taatacgactcactatagggagggcaggagtacttgtccaagg	Downstream region of *yddT*, fused with T7 promoter
yfjC-NB-fwd	cagggaatggccttgaagc	Upstream region of *yfjC*
yfjC-NB-rev	taatacgactcactatagggaggggacgaatgccatgtcagcc	Downstream region of *yfjC*, fused with T7 promoter
yitM-NB-fwd	gtcggttgtattggtgacgac	Upstream region of *yitM*
yitM-NB-rev	taatacgactcactatagggaggcaaccagcgttctgattgcc	Downstream region of *yitM*, fused with T7 promoter
yjhA-NB-fwd	cttgacgatcgggttagcgc	Upstream region of *yjhA*
yjhA-NB-rev	taatacgactcactatagggaggccccgatactagtgttcctccc	Downstream region of *yjhA*, fused with T7 promoter
yoaJ-NB-fwd	ccccgcaagcttcggcagc	Upstream region of *yoaJ*
yoaJ-NB-rev	taatacgactcactatagggaggggcccggtactgtataggc	Downstream region of *yoaJ*, fused with T7 promoter
yomL-NB-fwd	ggctgcatcattgactttaggc	Upstream region of *yomL*

yomL-NB-rev	taatacgactcactatagggagggcaggagtactagtccacgg	Downstream region of *yomL*, fused with T7 promoter
yqxI-NB-fwd	gttcttccgttggatggac	Upstream region of *yqxI*
yqxI-NB-rev	taatacgactcactatagggagggcaatagttctagctgtagaag	Downstream region of *yqxI*, fused with T7 promoter
yukC-NB-fwd	cgataaagggcttacgcc	Upstream region of *yukC*
yukC-NB-rev	taatacgactcactatagggagggcactccccaggttttcg	Downstream region of *yukC*, fused with T7 promoter
ywqH-NB-fwd	gcggatatcaaaagttcgctc	Upstream region of *ywqH*
ywqH-NB-rev	taatacgactcactatagggaggccgacttcttccgcgaactc	Downstream region of *ywqH*, fused with T7 promoter
yoaK-NB-fwd	gcagcagcttatcgaaacacc	Upstream region of *yoaK*
yoaK-NB-rev	taatacgactcactatagggaggccccgctcagtttcaacttagc	Downstream region of *yoaK*, fused with T7 promoter
cwlA-NB-fwd	gcgaatgatgcttcagcagcc	Upstream region of *cwlA*
cwlA-NB-rev	taatacgactcactatagggaggccggatggtagcgcatatgacg	Downstream region of *cwlA*, fused with T7 promoter
yozP-NB-fwd	ctgaaatgccatccattccag	Upstream region of *yozP*
yozP-NB-rev	taatacgactcactatagggagggttgaatctccagtactccacc	Downstream region of *yozP*, fused with T7 promoter
yddS-NB-fwd	gggggagcaggacttggagc	Upstream region of *yddS*
yddS-NB-rev	taatacgactcactatagggaggcaaacagcggatctggacgaag	Downstream region of *yddS*, fused with T7 promoter
yitN-NB-fwd	gtggacggaacggccgtc	Upstream region of *yitN*
yitN-NB-rev	taatacgactcactatagggaggctgaaacctctcccttcttgtg	Downstream region of *yitN*, fused with T7 promoter
yitO-NB-fwd	cagatgggtctgcattgcc	Upstream region of *yitO*
yitO-NB-rev	taatacgactcactatagggagggccattttctcggatcggtc	Downstream region of *yitO*, fused with T7 promoter
yfjB-NB-fwd	ggcgctcgtcactaagctgag	Upstream region of *yfjB*
yfjB-NB-rev	taatacgactcactatagggaggcggtcgctccggtaatcagtg	Downstream region of *yfjB*, fused with T7 promoter
yukE-NB-fwd	ggcacaggaggtaatgagg	Upstream region of *yukE*
yukE-NB-rev	taatacgactcactatagggagggtttgagctgctcgtattg	Downstream region of *yukE*, fused with T7 promoter
ywqJ-fwd	cccagcaagcagaagcaagacgg	Upstream region of *ywqJ*
ywqJ-rev	taatacgactcactatagggagggcttccgcttctcctcagacac	Downstream region of *ywqJ*, fused with T7 promoter
ybfJ-fwd	gcgggtgcagttcgcagacgc	Upstream region of *ybfJ*
ybfJ-rev	taatacgactcactatagggaggcgagatgtgatgggttactg	Downstream region of *ybfJ*, fused with T7 promoter

Tab. 5: Primers used for cloning in the pASK-IBA expression vectors

Name	Sequence (5'→3')	Description
degSptfwd	aaaaaaggtctcaaatgaataaaacaaagatggattcc	Forward primer for cloning of *degS* in pASK-IBA3 via *Bsa*I
degSptrev	aaaaaaggtctcagcgctaagagataacggaaccttaatc	Reverse primer for cloning of *degS* in pASK-IBA3 via *Bsa*I
degUptfwd	aaaaaaggtctcaagtgactaaagtaaacattg	Forward primer for cloning of *degU* in pASK-IBA3 via *Bsa*I
degUptrev	aaaaaaggtctcagcgcttctcatttctacccagcc	Reverse primer for cloning of *degU* in pASK-IBA3 via *Bsa*I

Tab. 6: Primers used for promoter regions cloning

Name	Sequence (5'→3')	Description
TreAfwdSmaI	tcccccgggcgccaataacataagccgagca	Cloning of *degS*-promoter region in pRB373 via *Sma*I
degSrevBamHI	cgcggatcccgttcgcgcaattgcttctc	For cloning of *degS*-promoter region in pRB373 via *BamH*I
TreAfwdSmaI	tcccccgggcgccaataacataagccgagca	Cloning of *degS*-promoter region in pJMB1 via *Sma*I
TreArevBamHI	cgcggatcccttttgttttattcatatttccctc	Cloning of *degS*-promoter region in pJMB1 via *BamH*I
degSshort-treAfwdSmaI	tcccccgggacagaggatcagaggctagcgc	Cloning of *degU*-promoter region in pJMB1 via *Sma*I
degSlong-treArevBamHI	cgcggatccaccttcacgaaataactgatggtcg	For cloning of *degS* gene and *degU*-promoter region in pJMB1 via *BamH*I
yddT_treAfwdSmaI	tcccccgggattctccaaggaaaaatgactc	Cloning of *yddT*-promoter region in pJMB1 via *Sma*I
yddT_treArevBamHI	cgcggatcctcttttctttctcaattgaaatcct	Cloning of *yddT*-promoter region in pJMB1 via *BamH*I
yitMNOP_treAfwdSmaI	tcccccgggggccgtccagtgaatcttccc	Cloning of *yitMNOP*-region or *yitP*-promoter in pJMB1 via *Sma*I
yitP_treArevBamHI	cgcggatcctatcgatttctgcattcgactgctc	Cloning of *yitP*-promoter in pJMB1 via *BamH*I
yitMNOP_treArevBamHI	cgcggatccactgaaacctctcccttcttgtgtt	Cloning of *yitMNOP*-region in pJMB1 via *BamH*I
yoaJ_treAfwdSmaI	tcccccggggctaagttgaaactgagcgggg	Cloning of *yoaJ*-promoter region in pJMB1 via *Sma*I
yoaJ_treArevBamHI	cgcggatccactcatgatctttttcatatttgg	Cloning of *yoaJ*-promoter region in pJMB1 via *BamH*I
yomL_treAfwdSmaI	tcccccgggtgccagagagttcgggttggac	Cloning of *yomL*-promoter region in pJMB1 via *Sma*I
yomL_treArevBamHI	cgcggatcctcttttctttctcaattgaaatcct	Cloning of *yomL*-promoter region in pJMB1 via *BamH*I
yqxIJ_treAfwdSmaI	tcccccgggcggcattgacggcgtgtatggt	Cloning of *yqxIJ*-region in pJMB1 via *Sma*I
yqxIJ_treArevBamHI	cgcggatcctcaaatcacctcaattaaaaagct	Cloning of *yqxIJ*-region in pJMB1 via *BamH*I

Tab. 7: Primers used for Primer extension analysis and sequencing

Name	Sequence (5'→3')	Description
degSPE2	ccctccgtcacggcgttgtc	5'region of *degS* used for Primer extension (DY-781)[a]
TreAfwd2	agcacatccacctgtagcg	Sequencing primer for pJMB1, upstream of *treA* gene
pJMB1rev2	aaaaaaggtctcaagtgactaaagtaaacattg	Sequencing primer for pJMB1, downstream of *treA* gene
pASK-IBA forward	gtgaaatgaatagttcgac	Sequencing primer for pASK-IBA3
pASK-IBA revers	cgcagtagcggtaaacggc	Sequencing primer for pASK-IBA3

[a]Oligonucleotides used for Primer extension analysis are purchased from biomers (Ulm). They are modified at their 5'region with the infrared dye DY-781.

4. Growth media and cultivation conditions

4.1. Complex media

E. coli strains were generally cultivated in LB (Luria Bertani) medium (Sambrook 2001). For solid medium 15g agar per 1 liter medium was added. If necessary the medium was additionally supplemented with antibiotics.

4.2. Minimal media

All *B. subtilis* strains were grown either in Spizizen's minimal medium (SMM) (Spizizen, 1958) or in Helmann minimal medium described by Chen et al., 1993. After autoclaving the SMM was supplemented with the following additives: 0.5% glucose as a carbon source, trace elements (Haarwood & Cutting, 1990) and the amino acids L-tryptophan (20mg/l) and L-phenylalanine (18 mg/l). In the case of strain 168 and its derivatives only L-tryptophan was added.

The components for the Helmann minimal medium are listed below.

Tab. 8: Helmann MM for *B. subtilis*

Component	Final concentration in medium	Trace elements	Final concentration in medium
$FeCI_3$	5 µM	$(NH_4)_6Mo_7O_{24}$	3 nM
MOPS	40 mM	H_3BO_3	400 nM
$(NH_4)_2SO_4$	15 mM	$CoCI_2$	30 nM
$MgSO_4$	1.7 mM	$CuSO_4$	10 nM

Sodium citrate	3.5 mM	$ZnSO_4$	10 nM
Potassium glutamate	5 mM	$MnCI_2$	80 nM
Glucose	2% (w/v)		
Potasium phosphate	2 mM		
Tryptophan	80 mg/l		
Phenylalanine	100 mg/l		
Leucine	50 mg/l		

The pH values of potassium morpholinopropane sulfate (MOPS) and potassium phosphate were adjusted to 7.4 with KOH, and to 7.0 respectively. Leucine was added only in the case of strain PB5246. All components were mixed together prior to cultivation and the medium was stored up to 3 days at RT.

4.3. Transformation medium

For the transformation of *B. subtilis* was used LS (low salt) medium, freshly prepared prior to use.

H_2O	17.2 ml
SMM-medium (5x)	2 ml
20% Glucose	0.2 ml
L-Trp (5mg/ml)	20 µl
L-Phe (3mg/ml)	30 µl
2% Casein-hydrolisate	0.1 ml
10% Yeast extract	0.2 ml
50mM Spermin	0.2 ml
1M $MgCI_2$	50 µl

4.4 Compatible solutes and antibiotics

If necessary, to the autoclaved and cooled to 50°C media were added certain antibiotics and compatible solutes from sterile stock solutions. These are listed below.

Tab. 9: Compatible solutes and antibiotics

Substance	**Solvent**	**Final concentration for *B. subtilis***	**Final concentration for *E. coli***
Ampicillin	H_2O	-	100 µg/ml
Kanamycin	H_2O	5 µg/ml	-
Chloramphenycol	Ethanol	5 µg/ml	30 µg/ml
Erythromycin	H_2O	1 µg/ml	-
Lyncomicin	H_2O	15 µg/ml	-
Glycine betaine	H_2O	1 mM	-

4.5. Sterilization

All media were sterilized for 20 min at 121°C and 1 bar overpressure. The heat sensitive components like antibiotics, sugars and amino acids were sterile filtrated. For all glass instruments 4 hours at 180°C were applied.

4.6. Determination of the osmolarity

Determination of the osmolarity of various buffers and growth media was performed in a vapour pressure osmometer (model 5.500; Wescor).

4.7. Cultivation conditions

All cultures were routinely grown under aerobic conditions at 37°C. The agar plates were cultivated in an incubator and the liquid cultures in a test-tube roller, water bath or air shaker at 220 rpm. Small volumes up to 5 ml were incubated in test-tubes. Erlenmeyer flasks with total volumes exceeding those of the contained media by about 5-fold were used for growth of the cultures.

B. subtilis

For all applied experiments *B. subtilis* precultures were incubated as follows: 5 ml of the certain medium were inoculated with single colony from an agar plate and incubated over day on a test-tube roller. This well grown culture was then used for inoculation of 20 ml overnight preculture. The latter was used on the next day for the main culture inoculation. For the experiments where higher salt concentrations were applied, the medium for the main culture was maintained so, that after inoculation to reach the desired osmolarity.

For the growth monitoring and RNA isolation, overnight cultures with OD_{578} of between 1 and 2 were used for inoculation of 100ml main culture in 500ml Erlenmeyer flasks and starting OD_{578} of 0.1. For the TreA activity measurements (section 6.6.), smaller culture volumes were used, namely 20 ml. For the HPLC analysis 100 ml precultures were used to inoculate 1 liter main culture in 5l flasks and incubated in an air shaker.

E. coli

Culture volume of 5 ml was incubated on a test-tube roller overnight at 37°C. Bigger volumes from 20 to 200 ml were incubated in Erlenmeyer flasks in water bath or air shaker set at 220rpm. For heterologous synthesis of proteins, *E.coli* BL21 cells were cultivated from a single colony overnight in 100 ml LB medium at 30°C in an air shaker. Subsequently, the cultures were transferred into a fresh 1 liter LB medium with starting OD_{578} of 0.1 at 25 or 30°C.

The culture growth was monitored by photometric measurements of the optical density at wavelength of 578 nm (OD_{578}) with a spectrophotometer (Ultraspec 3100, Amersham Bioscinces, Freiburg) using pure medium as a blank.

5. Molecular biology approaches

5. 1. Agarose gel electrophoresis

Gel electrophoresis of DNA was performed using 1% agarose gels (w/v) in 1X TAE buffer as described by Sambrook et al. (2001). The DNA samples were mixed with 6X Loading Dye (0.2% Bromphenolblue, 0.2% Xylencyanol, dissolved in 50% glycerine) and the gels were run for 1-2 h at 70 to 110 V. A *Bst*EII digested λ phage DNA (Fermentas, St. Leon-Rot) was used as a standard for the fragment size. For DNA visualization, the gels were stained in ethidium bromide (1 µg/ml) for 10-15 min and exposed to UV light. Pictures were taken for documentation with a video camera from INTAS (Göttingen).

1X TAE buffer: 40 mM Tris, 1 mM EDTA, pH (acetic acid) 8.0

5.2. DNA techniques

5.2.1. Isolation of genomic DNA

The genomic DNA from *B. subtilis* was isolated by using QIAGEN Genomic-tip 20 kit. The *B. subtilis* cells were incubated in 20 ml SMM medium at 37°C. When reached OD_{578} of 0.8 to 1.0, 14 ml of the culture were harvested via centrifugation (10 min, 5000 rpm, 4°C), and the genomic DNA was isolated according to the supplier instructions.

5.2.2. Isolation of plasmid DNA

The isolation of plasmid DNA was performed following the principle of alkaline lysis (Birnboim and Doly, 1979). For this purpose, *E. coli* cells were cultivated as described in section 4.6. For the isolation of plasmid DNA from these cultures, the QIAGEN Plasmid Midi Kit was used as recommended by the supplier.

5.2.3. Polymerase chain reaction (PCR)

The *in vitro* amplification of specific DNA fragments was performed by the polymerase chain reaction (PCR) (Mullis et al., 1986) using the Easy Start Kit 100 (Molecular Bio Products) as recommended by the supplier. The primers used for the reaction were diluted to a concentration of 10 pmol/µl. As a template served genomic DNA, plasmid DNA or DNA fragments. The reactions were performed with Taq-DNA-Polymerase (Eppendorf, Hamburg), Phusion or DyNAzyme polymerases (Finnzymes) in the "Trio-Thermoblock-Cycle" (Biometra, Göttingen). All PCR products were purified using the QIAquick PCR Purification Kit according to the supplier's instructions.

5.2.4. Determination of DNA concentration

The DNA concentration was estimated photometrically with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington USA).

5.2.5. Digestion and ligation of DNA

The digestion of DNA with restriction endonucleases was performed in 20-50 µl total volume with 0.5 to 1 µg DNA sample. The appropriate buffers, concentration of the corresponding endonucleases, temperature and time of exposure were followed as recommended from the supplier (Fermentas, St. Leon-Rot or Amersham Pharmacia, Freiburg). If dephosphorylation

of 5' ends of the vector DNA was necessary, 1 µl (1U/µl) calf intestinal alkaline phosphatase (CIAP, Fermentas) was added to the samples.

After restriction and/or dephosphorylation, the DNA samples were purified with the QIAquick PCR Purification Kit following the supplier's instructions and visualized on an agarose gel as described in section 5.1.

For ligation of the DNA fragments into restricted vectors, each ligation mixture was setup in the following way:

200 ng of vector DNA
600 ng of insert DNA
2 µl 10X ligase buffer (Amersham)
2 µl T4 ligase (Amersham)
x µl H_2O

The mixture was brought to 20 µl with nuclease-free water, incubated overnight at 16°C in water bath and used subsequently for transformation.

5.2.6. DNA sequencing

DNA sequence analysis (PCR products and vectors) were performed by MWG-Biotech, Martinsried. Standard vector-derived primers were used for the complete sequencing of DNA templates. All samples were double-strand sequenced.

The manual sequencing reaction that was run along with the primer extension reaction (section 5.3.6.) of the *degS*-promoter region was performed according to the dideoxy chain termination method (Sanger et al., 1977). For this purpose, a CycleReader^{TM} Auto DNA Sequencing Kit (Fermentas, St. Leon-Rot) was used. For the sequence reaction the template DNA was mixed with a 5'-DY 781-labelled primer and subsequently visualized with LI-COR sequencer model 4200 (LI-COR Biosciences, Bad Homburg). The data were analysed with an E-Seq software.

5.3. RNA techniques

5.3.1. Isolation of total RNA from *B.subtilis*

For the isolation of total RNA from *B.subtlis*, the cells were cultivated in Helmann medium as described in section 4.6. When reached OD_{578} of 0.8-1, 20 ml of the culture were mixed with equal volume of ice-cold killing buffer (20 mM Tris-HCI, pH 7.5, 5 mM $MgCI_2$, 20 mM NaN_3). The cells were collected by centrifugation (5000 rpm, 10 min, 4°C), the resulting

pellet was resuspended in 1 ml killing buffer and transferred in an Eppendorf tube. After another centrifugation step (13000rpm, 5 min, RT) the cells were immediately frozen in liquid nitrogen and stored at -80°C until use.

For the cell lysis, the bacterial pellet was resuspended in 200 µl killing buffer and transferred into a disruption ball. The cells were opened mechanically in a cell disruptor (2 min, 2600 rpm). The resulting powder was resuspended in 4 ml prewarmed at 50°C lysis buffer (4M Guanidinium thiocyanate, 0.025M Sodium acetate pH 5.2, 0.5% N-lauroylsarcosinate), 1 ml aliquots were transferred in Eppendorf tubes and snap-frozen in liquid nitrogen.

For the RNA isolation, the cell lysate was mixed with one volume of acid phenol solution (Aqua-phenol/chloroform/isoamylalcohol as 25:24:1), shaked on an Eppendorf Thermomixer for 15 min at RT and spun down for 8 min in a table top centrifuge at high speed. The supernatant (850 µl) was transferred into a new Eppendorf tube and the phenol extraction step was repeated. The resulting aqueous phase (700 µl) was mix with one volume of chlorophorm-isoamylalcohol (24:1) solution, shaken for 5 min at RT and spun down for another 5 min. After separation of the two phases, the upper one was once again transferred into a fresh Eppendorf tube and mixed with 1/10 volume of 3M sodium acetate pH 5.2. The RNA was precipitated by the addition of 1 ml 100% isopropanol overnight at -20°C. After centrifugation for 20 min at 15000 rpm (4°C) the RNA pellet was washed with 70% Ethanol and dissolved in 30 µl DEPC-treated water.

Besides, total RNA was isolated with the "High Pure RNA Isolation Kit" (Roche, Mannheim) in accordance to the supplier's instructions.

5.3.2. Determination of RNA concentration and purity

For the Northern blot and dot blot analysis the RNA concentration was estimated photometrically with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington USA).

The integrity of the isolated total RNA was estimated by gel electrophoresis. For this purpose 2 µg of total RNA were mixed with an equal volume of RNA loading buffer (peqlab GmbH, Erlangen) and denatured at 70°C for 10 min. Subsequently, the samples were kept on ice for 2 min and loaded on 1% agarose gel. After staining in ethidium bromide and UV visualization, the two bands of rRNA could be seen.

5.3.3. Synthesis of digoxigenin-labelled RNA probes

Digoxigenin-labelled RNA probes were produced by *in vitro* transcription with the Strip-EZ® RNA T7 Kit (Ambion, Austin, TX, USA). The DNA template for the *in vitro* transcription was generated via PCR of the responding gene region. In addition the sequence of the T7-promoter was introduced in the PCR product through one of the primers (Tab.4) and oriented so, that an antisense-RNA to be transcribed. To verify that PCR products were unique and had the expected size, they were examined on an agarose gel before use. The RNA probes were labelled by incorporation of the digoxigenin-11-UTP (Roche, Mannheim) in the reaction. After synthesis of the antisense-RNA probe, the DNA template was removed through DNase treatment and the probe was stored at -20°C until use.

5.3.4. Northern blot analysis

The expression patterns of specific genes were analyzed by Northern blot analysis, where the size of an mRNA transcript could be determined as well. For this purpose 5-10 µg of total RNA were mixed with equal volume of RNA Loading buffer (peqlab GmbH) and denatured for 10 min at 70°C. In parallel, 6 µl of peqGOLD High Range RNA Ladder (peqlab GmbH) were treated in the same manner and used as a size marker. After the denaturation step, the samples were immediately chilled on ice and the RNA was separated by means of 1.4% agarose-MOPS gel electrophoresis. The gel run for 3 hours at 70 V and 1X MOPS buffer as a running buffer. In the next step the gel was placed on the top of a 10X SSC-equilibrated nylon membrane (Nytran 0,45; Schleicher & Schuell BioScience, Dassel). The RNA transfer was performed via pressure overnight in 10X SSC buffer. Subsequently the migrated RNA molecules were crosslinked by the exposure to a UV light (Stratalinker®, Stratagene). To visualize the molecular weight marker, the corresponding part of the membrane was cut out and dyed with methylene blue.

The membrane was placed in a hybridization bottle with 10-15 ml hybridization solution. The pre-hybridization step was performed for 4 hours at 68°C with gentle agitation in a rotary hybridization oven.

The hybridization step was performed by the addition of denatured DIG-labelled RNA probe (section 5.3.3.), specific for the transcript of interest, to the hybridization solution. The denaturation of the probe was achieved at 95°C for 5 min prior to use. The hybridization was performed overnight at 68°C. Afterwards the membrane was washed twice in 20 ml wash buffer 1 for 5 min at 68°C and two times in 20 ml wash buffer 2 for 15 min at 68°C. To prevent non-specific binding reactions, the membrane was incubated for 30 min at RT in

20 ml blocking buffer. Afterwards 1 µl alkaline phosphatase conjugated anti-digoxigenin Fab fragments (Roche, Mannheim) was added and the membrane was incubated for another 30 min at RT. After several washing steps in wash buffer 3 for 30 min each, the membrane was equilibrated in 100 ml 0.1M Tris-HCI, pH 9.5. The RNA transcripts were detected using a Phosphor Imager (Storm 860, Amersham Biosciences, Freiburg) with 0.5 ml ECF-Vista (Amersham Biosciences, Freiburg) as a substrate.

1X MOPS buffer: 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0
10X SSC: 1.5 M NaCI, 0.15 M sodium citrate
Methylene blue dye: 0.1% (w/v) methyleneblue, 300 mM sodium acetate, pH 5.6
Hybridization solution: 50% formamide, 5X SSC, 0.1% N-lauroylsarcosinate, 0.02% SDS, 2% blocking reagent (Roche, Mannheim)
Wash buffer 1: 2X SSC, 0.1% SDS
Wash buffer 2: 0.2% SSC, 0.% SDS
Wash buffer 3: 100 mM maleic acid, 150 mM NaCI, pH 7.5
Blocking buffer: 1% blocking reagent in wash buffer 3

5.3.5. Dot blot

Another technique for studying the specific gene expression patterns was the dot blot. For the preparation of RNA dot blots, 1.5 µg of total RNA was mixed with 100 µL 20X SSC and spotted onto a nylon membrane (Nytran 0,45; Schleicher & Schuell BioScience, Dassel), which was pre-equilibrated in 20X SSC, using the Dot Blotter (Schleicher & Schuell BioScience, Dassel). After washing several times with 20X SSC buffer and drying, the membrane was crosslinked by UV radiation (Stratalinker®, Stratagene). Next steps of washing and detection of the signal followed the same procedure as for Northern blot analysis.

5.3.6. Primer extension analysis

Primer extension analysis was performed for determination of the exact start site for transcription of a certain gene. After verifying the integrity of the isolated total RNA, 25 µg of it were incubated together with 2.5 pmol of a specific labelled primer (Tab.7) and 4 µl 10X AMV Reverse Transcription buffer (Promega, Mannheim) for 3 min at 75°C. After the annealing step, the samples were slowly cool down to 42°C and the following components for the reverse transcription reaction were added: 1 µl dNTP mix (8 mM), 1 µl AMV Reverse Transcriptase (Promega), 1µl Recombinant RNasin® Ribonuclease Inhibitor (Promega),

0.4 µl DTT (100mM). The reaction mix was further incubated at 42°C for 1 hour. The synthesized cDNA was precipitated overnight by the addition of 500 µl 100% ice-cold ethanol and 1 µl glycogen (10mg/ml), and the resulting pellet was resuspended in 10 µl formamide solution (1X Stop solution from "CycleReader™ Auto DNA Sequencing Kit", Fermentas). The sample was stored at -20°C till use. Before loading on a sequencing gel, the cDNA was denatured by heating for 10 min at 95°C and immediately placed on ice.

The primer extension products were analysed on a 25 x 40 cm denaturing polyacrylamide gel. The latter was prepared from Sequagel XR ultra pure and Sequagel complete ultra pure (Biozym) following the manufacturer's instructions.

In order to estimate the exact transcription start point, a sequence reaction (section 5.2.6.) of the region of interest was loaded next to the primer extension on the gel. For this purpose the same fluorescently labelled primer was used along with the corresponding promoter region. The data were analysed with an E-Seq software.

5.4. Bacterial transformation

5.4.1. Preparation of competent *B. subtilis* cells and transformation

For transformation of *B. subtilis* cells a slightly modified "High salt / Low salt"- method (Harwood & Cutting, 1990) was applied. For preparation of competent cells, 20 ml of SMM medium were inoculated with *B. subtilis* cells and cultivated until OD_{578} of 0.8-1. This exponentially grown culture was used to inoculate 20 ml LS medium and the cells were incubated in water bath for 3h at 30°C and 100 rpm.

The naturally competent cells were then transformed with plasmid or chromosomal DNA, respectively, and further incubated for 2h at 37°C and 1400 rpm on an Eppendorf Thermomixer (Eppendorf GmbH). Subsequently, the cell suspension was plated on LB-plates with the appropriate antibiotic.

LS medium: 1X Base (1X SMM without trace elements), 0.5 % Glucose, Tryptophan (5 µg/ml), 0.01% Casein Hydrolysate, 0.1% Yeast extract, 0.5 mM Spermin, 2.5 mM $MgCI_2$

Besides, *B. subtilis* transformation was performed by the use of "TransformAid™ Bacterial Transformation Kit" (Fermentas, St. Leon-Rot) in accordance to the supplier's instructions.

5.4.2. Preparation of competent *E. coli* cells and transformation

The preparation of electrocompetent *E. coli* cells was established according to Ausubel et. al., 1994. When the transformation was performed with ligation samples, the latter were dialysed

against ddH_2O in order to remove any salt traces. For this purpose 2.5-5 µl of ligation reaction were pipetted onto a membrane filter disks with pore size of 0.02 µm (Millipore, Schwalbach) and incubated for 30-60 min at RT.

For the transformation, 50 µl of electrocompetent cells were thawed on ice for 1-2 min and the plasmid DNA was added. The electroporation of ligations and plasmid preparations was performed in a MicroPulser™ (BioRad, Munich) according to the supplier's instructions. In the following step the transformed cells were incubated in 1 ml LB medium for 1h at 37°C and 1400 rpm in an Eppendorf Thermomixer (Eppendorf GmbH). Subsequently, the cell suspension was plated on LB-plates with the appropriate antibiotic.

5.5. Construction of plasmids and bacterial strains

5.5.1. Plasmid construction

Construction of the plasmids listed in Table 3 is described below. For the PCR amplification chromosomal DNA from *B. subtilis* wild type strain (168 or JH642) was used as a template. The primers applied for the construction are listed in 5´- to 3´- end direction.

pTH1

This plasmid is a pJMB1 derivative and was constructed in the following way. The *degS* promoter region was amplified using the primers TreAfwdSmaI and TreArevBamHI. The amplified promoter region was digested with *BamH*I and *Sma*I, and cloned upstream of the *treA* coding region into pJMB1 digested with the same endonucleases. The corresponding clones were selected on LB/Amp plates. The plasmids were then screened to check for those with the correct orientation and subsequently introduced into the chromosome of *B. subtilis*.

pTH10

Plasmid pTH10 was used for cloning the *yddT* promoter region upstream of the *treA* coding sequence in pJMB1. The region of interest was amplified in a PCR using the primers yddT_treAfwdSmaI and yddT_treArevBamHI. Afterwards the same cloning strategy as for the pTH1 was followed.

pTH11

Plasmid pTH11 is a pJMB1 derivative that carries *amyE::[Φ(yitMNOP'-treA)1 cat]* construct. The same cloning strategy as for pTH1 was followed.

pTH12

A pJMB1 derivative plasmid that carries *amyE::[Φ(yitP'-treA)1 cat]* construct. The same cloning strategy as for pTH1 was followed.

pTH13

A pJMB1 derivative plasmid that carries *amyE::[Φ(yoaJ'-treA)1 cat]* construct. The same cloning strategy as for pTH1 was followed.

pTH14

A pJMB1 derivative plasmid that carries *amyE::[Φ(yomL'-treA)1 cat]* construct. The same cloning strategy as for pTH1 was followed.

pTH15

A pJMB1 derivative plasmid that carries *amyE::[Φ(yqxIJ'-treA)1 cat]* construct. The same cloning strategy as for pTH1 was followed.

pTH16

A pJMB1 derivative plasmid that carries *amyE::[Φ(degSU'-treA)1 cat]* construct. The same cloning strategy as for pTH1 was followed.

pTH17

A pJMB1 derivative plasmid that carries *amyE::[Φ(degU'-treA)1 cat]* construct. The same cloning strategy as for pTH1 was followed.

pTH2

The *degS* promoter region was amplified via PCR using the primers TreAfwdSmaI and TreArevBamHI that contain *SmaI* and *BamH*I restriction sites respectively. The resulting product was cloned into *E. coli* - *B. subtilis* shuttle vector pRB373, digested with the same endonucleases. The positive clones were selected on LB/Kan plates and verified by sequencing.

pTH3

This expression plasmid was used to introduce the *degS* gene under the control of the tetracycline inducible promoter of pASK-IBA3. For the purpose the *degS* coding region was amplified by PCR using the primers degSptfwd and degSptrev. The resulted product was then cloned into the *Bsa*I site of pASK-IBA3 using the *Bsa*I sites located in both primers. The orientation of the *degS* gene leads to the expression of recombinant protein with Strep-tag II fused to its C-terminus. The correct orientation of the insert was further verified by sequencing of the vector.

pTH4

This expression plasmid was used for the overproduction of the DegU protein. For this purpose, the coding region of *degU* was cloned in pASK-IBA3 under the transcriptional control of tetracycline promoter in the same manner as for the *degS*. This resulted in a construct leading to expression of a recombinant protein bearing a Strep-tag II fused to its C-

terminus. The correct orientation of the insert was further verified by sequencing of the vector.

5.5.2. Strain construction

All *treA*-reporter gene fusion constructs are derivatives from pJMB1. This plasmid carries the chloramphenicol resistance determinant and a promotorless *treA* gene between two fragments of the *B. subtilis amyE* gene. This allows the integration of the constructed fusions in the chromosomal *amyE* locus of *B. subtilis* via homologue recombination. For this purpose a *B. subtilis* strain with knocked-out *treA* gene was transformed with the respective plasmids. The resulted transformants were selected on chloramphenicol and the AmyE$^-$ phenotype was verified in an amylase assay (section 6.7.).

THB1

For the construction of THB1{168 Δ(*treA*::*neo*) *amyE::[Φ(degS'-treA)1*] *cat*} competent cells from *B. subtilis* GSB4 [168 (*treA*::*neo*)] were transformed with *Xho*I and *Pst*I digested pTH1.

THB2

Analogous to THB1. GSB4-competent cells were transformed with pJMB1

THB4

For the construction of THB4 [168 Δ(*treA*::*erm*)], competent cells from B. subtilis 168 were transformed with chromosomal DNA isolated from JGB34.

THB41

For the construction of THB41 [168 Δ(*treA*::*erm*) *amyE::[Φ(degS'-treA)1 cat*], competent cells from THB4 were transformed with *Xho*I and *Pst*I digested pTH1.

THB42: Analogous to THB41. THB4 competent cells were transformed with pJMB1

THB410: Analogous to THB41. THB4 competent cells were transformed with pTH10

THB411: Analogous to THB41. THB4 competent cells were transformed with pTH11 x *Xho*I

THB412: Analogous to THB41. THB4 competent cells were transformed with pTH12

THB413: Analogous to THB41. THB4 competent cells were transformed with pTH13

THB414: Analogous to THB41. THB4 competent cells were transformed with pTH14

THB415: Analogous to THB41. THB4 competent cells were transformed with pTH15

THB416: Analogous to THB41. THB4 competent cells were transformed with pTH16 x *Xho*I

THB417: Analogous to THB41. THB4 competent cells were transformed with pTH17

THB21: THB41 cells were transformed with chromosomal DNA from MD282

THB22: THB42 cells were transformed with chromosomal DNA from MD282

THB210: THB410 cells were transformed with chromosomal DNA from MD282

THB211: THB411 cells were transformed with chromosomal DNA from MD282

THB212: THB412 cells were transformed with chromosomal DNA from MD282

THB213: THB413 cells were transformed with chromosomal DNA from MD282

THB214: THB414 cells were transformed with chromosomal DNA from MD282

THB215: THB415 cells were transformed with chromosomal DNA from MD282

THB216: THB416 cells were transformed with chromosomal DNA from MD282

THB217: THB417 cells were transformed with chromosomal DNA from MD282

THB31: THB41 cells were transformed with chromosomal DNA from MD300

THB32: THB42 cells were transformed with chromosomal DNA from MD300

THB310: THB410 cells were transformed with chromosomal DNA from MD300

THB311: THB411 cells were transformed with chromosomal DNA from MD300

THB312: THB412 cells were transformed with chromosomal DNA from MD300

THB313: THB413 cells were transformed with chromosomal DNA from MD300

THB314: THB414 cells were transformed with chromosomal DNA from MD300

THB315: THB415 cells were transformed with chromosomal DNA from MD300

THB316: THB416 cells were transformed with chromosomal DNA from MD300

THB317: THB417 cells were transformed with chromosomal DNA from MD300

THB253

For the construction of THB253 {JH642 *(treA*::*neo*)1 *amyE*::[Φ(*degS*'-*treA)1 cat*]} competent cells from *B. subtilis* FSB1 [JH642*(treA::neo)1*] were transformed with *Xho*I and *Pst*I digested pTH1.

THB282

JH642 competent cells were transformed with chromosomal DNA from MD282

THB300

JH642 competent cells were transformed with chromosomal DNA from MD300

6. Biochemical approaches

6.1. SDS Polyacrylamide Gel Electrophoresis (PAGE)

To analyse the overexpression patterns and purity of proteins under denaturing conditions, a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was applied (Laemmli, 1970). The protein separation was achieved by the use of stacking and resolving gel that are listed below. To estimate the size of the proteins the Unstained Protein Molecular Weight Marker or PageRuler^{TM} Prestained Protein Ladder (Fermentas, St. Leon-Rot) were

used. The protein samples were mixed with equal volume of 2X SDS sample buffer and heated at 95°C for 5 min prior loading the gel. The electrophoretic run was performed in 1X Tris-glycine-SDS running buffer in a Bio-Rad electrophoresis apparatus (Bio-Rad, Munich) at 35-40 mA electric current. When the Bromphenol Blue dye front moved through the bottom of the resolving gel, the latter was disassembled and stained in Coomassie Brilliant Blue for visualisation of the separated proteins. The gel was soaked for 15 min in staining solution and then washed with destaining solution until the protein bands became clear.

15% separation gel: 5 ml acrylamide: bisacrylamide (37.5:1), 2.5 ml 1.5M Tris-HCL (pH 8.8) with 0.1% SDS, 2.5 ml H_2O, 20 µl TEMED, 40 µl 10% APS
Stacking gel: 1 ml acrylamide: bisacrylamide (37.5:1), 2.5 ml 1.5M Tris-HCL (pH 8.8) with 0.1% SDS, 6.5 ml H_2O, 20 µl TEMED, 40 µl 10% APS
SDS Running buffer: 50 mM Tris, 384 mM glycine, 0.1% SDS
SDS sample buffer (1X): 62.5 mM Tris-HCI pH 6.8, 2% SDS (w/v), 10% glycerol, 10 mM DTT, 0.002% Bromphenol Blue
Staining solution: 0.25% (w/v) Coomassie Brilliant Blue, 25% (v/v) isopropanol, 10% (v/v) acetic acid
Destaining solution: 28% (v/v) isopropanol, 5% (v/v) acetic acid

6.2. Determination of protein concentration

For the quantification of total protein the BCA™ Protein Assay Kit (Pierce, Rockford) was used. This method is based on the reduction of Cu^{+2} to Cu^{+1} by a protein in an alkaline medium (the biuret reaction) and subsequent colorimetric detection of the Cu^{+1} using a reagent containing bicinchoninic acid (BCA). The test was performed as recommended by the supplier and the protein samples were measured at 562 nm wavelength.
Alternatively, the protein concentration was measured photometrically with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington USA) according to the manufacturer's instructions.

6.3. Heterologous overexpression and purification of DegS and DegU proteins from *B. subtilis*

6.3.1. Heterologous expression of DegS

For the overexpression of DegS protein were used *E. coli* BL21 cells harbouring the plasmid pOFXtac-SL2. This plasmid contains *groES-groEL* genes under the control of IPTG-

inducible P_{tac}-promoter. These chaperone expressing cells were used for transformation with pTH3, a pASK-IBA3 derivative vector containing the *degS* coding region from *B. subtilis*. This strain was used to isolate DegS, fused to a Strep-tag at its C-terminus. The resulting culture was incubated in 2 X 1 liter LB medium with ampicillin (100 µg/l) and chloramphenicol (30 µg/l) at 30°C until mid-exponential phase (OD_{578} of 0.5). The induction was carried out by the addition of 1 mM IPTG and 200 µg/l AHT. After 1-2 hours (OD_{578} of 0.9-1.0) the cells were harvested and stored at -20°C for subsequent use. The cultures were grown aerobically and aerated by constant shaking (220 rpm). The total protein content was monitored via SDS-PAGE.

6.3.2. Heterologous expression of DegU

For the heterologous synthesis of DegU, *E. coli* BL21 cells were transformed with pTH4. This plasmid is pASK-IBA3 derivative containing the *degU* coding region from *B. subtilis* in the *Bsa*I restriction site. This strain was used to isolate DegU, fused to a Strep-tag at its C-terminus. The overexpression of DegU was performed in 2 X 1 liter LB medium supplemented with 100 µg/ml ampicillin. The culture was inoculated with starting OD_{578} of 0.1 and further incubation at 30°C. After reaching an OD_{578} of 0.5, the heterologous gene expression was induced by the addition of 40 to 200 µg/l anhydrotetracycline (AHT). The culture was further incubated for 30-60 min until OD_{578} of 0.9-1.0, the cells were harvested and stored at -20°C till use. The cultures were grown aerobically and aerated by constant shaking (220 rpm). The overproduction of the protein was monitored via SDS-PAGE.

6.3.3. Purification of DegS and DegU

The pASK-IBA3-derivatives that carry DegS and DegU respectively, drive the overexpression of the recombinant proteins in the cytoplasm of *E. coli* cells. For this purpose, the cell pellets with overproduced proteins were thawed on ice, resuspended in 10-15 ml lysis buffer and disrupted by three passages through a French Pressure Cell Press. The cell debris and membranes were removed by ultracentrifugation (1 h, 100 000 x g, 4°C) and the supernatant fraction containing the respective solubilized protein was filtrated (0.45 µm) and purified by affinity chromatography via FPLC equipment (Amersham Biosciences) using a 5 ml of Strep-Tactin Superflow column (IBA, Göttingen). The column was equilibrated with 5 column bed volumes of buffer W (washing buffer) and the supernatant was loaded to allow binding of the proteins to the StrepTactin material. The chromatography was performed at 4°C with flow rate of 1 ml/min. The column was further washed with 24 volumes (120 ml)

buffer W and the target fusion protein was eluted with 30 ml buffer E. The eluate was collected in 5 ml fractions and subjected to SDS-PAGE for monitoring the purity of the DegS and DegU proteins. The concentration of the purified fusion proteins was measured and they were stored at 4°C.

Lysis buffer: 5% glycerol (v/v), 50 mM Tris-HCI pH 7.5-8.0, 50 mM NaCI, 2 mM DTT, 0.5 mM PMSF, 0.5 mM benzamidine, 0.5 mM EDTA, 0.08% lysozyme.
Buffer W: 100 mM Tris-HCI pH 8.0, 150 mM NaCI, 1 mM EDTA
Buffer E: buffer W containing 2.5 mM desthiobiotin

6.4. Phosphorylation assay

If otherwise indicated, all phosphorylation reactions were performed at 37°C. Phosphorylated proteins were detected by Phosphorimager (Storm 860, Amersham Biosciences, Freiburg). Quantification was performed by image analysis with Image Quant^{TM}-Software of Molecular Dynamics.

6.4.1. Autophosphorylation of DegS sensor kinase

To test the autophosphorylation activity of DegS, 1-1.5 µM of the sensor kinase were incubated in phosphorylation buffer containing 50 mM Tris-HCI pH 8.0, 10% (v/v) glycerol, 2 mM DTT, 50 mM KCI and 0.5 mM EDTA. If indicated, the autophosphorylation was determined in the presence of various solutes. Phosphorylation reaction was initiated by addition of 20 µM [γ-^{32}]ATP (2.38 Ci/mmol). At the indicated time points, 16 µl aliquots were removed and the reaction was stopped by the addition of 4 µl 5X SDS sample buffer. All samples were immediately subjected to SDS-polyacrylamide gel electrophoresis. Shortly before stopping the SDS-PAGE, 2.1 pmol of [γ-^{32}]ATP standard were loaded on the gel. Subsequently the gels were dried and exposed to a storage phosphor imaging plate. Phosphorylation of the protein was analyzed as a phosphorimage and quantified.

SDS sample buffer (5X): 225 mM Tris-HCI pH 6.8, 50% (v/v) glycerol, 5% (w/v) SDS, 250 mM DTT, 0.05% Bromphenol Blue

6.4.2. Phosphotransfer from the DegS sensor kinase to the DegU regulator

To analyze the phosphotrasfer from DegS kinase to its response regulator DegU, 1-1.5 µM of the sensor kinase were incubated in phosphorylation buffer containing 50 mM Tris-HCI pH 8.0, 10% (v/v) glycerol, 2 mM DTT, 50 mM KCI and 0.5 mM EDTA. The DegS

autophosphorylation was initiated by addition of 20 µM [γ-^{32}]ATP (2.38 Ci/mmol). After incubation for 20 min, an aliquote was taken out and the reaction was stopped by mixing with 5X SDS sample buffer. Then purified DegU was added to the reaction mixture with DegS:DegU ratio ranging from 1:13 to 1:20 and the incubation was continued. Further aliquots were removed at the indicated time points and mixed with 5X SDS sample buffer. All samples were immediately subjected to SDS-polyacrylamide gel electrophoresis. Shortly before stopping the SDS-PAGE, 2.1 pmol of [γ-^{32}]ATP standard were loaded on the gel. After drying the gel was exposed to a storage phosphor imaging plate. Phosphorylation of the proteins was analyzed as a phosphorimage and quantified.

6.5. HPLC

HPLC analysis was performed with an HPLC system from Sykam. The HPLC system was programmed with run time of 40 min, column temeperature of 45°C and a two-buffer gradient elution system.

6.5.1. Extraction according to Bligh and Dyer

Cells for high-performance liquid chromatography (HPLC) analysis of glutamate were extracted using a modified Bligh and Dyer technique (Bligh & Dyer, 1959; Kunte et al., 1993). For this purpose *B. subtilis* cultures were incubated at 37°C on a rotary shaker (220 rpm) and upon reaching the OD_{578} of 2.0 the cells were subjected to 0.4M salt shock by the addition of NaCI. The incubation was continued and 20 ml aliquots were taken at the indicated time points. The cells were washed twice in isotonic medium and harvested by centrifugation. The resulting pellets were immediately frozen in liquid nitrogen and subjected to lyophylisation (Christ Alpha 1-4-liophylisator, B. Braun, Biotech International) for 48 hours. The resulted dry cell mass was determined and the cells were extracted with 500 µl extract solution [methanol/chloroform/water, 10:5:4 (v/v/v)] by incubation for 30 min in a sonication bath and subsequent shaking (Vortex Genie 2, Scientific Industries) at maximal rate for 30 min at RT. Then equal volumes (130 µl) of chloroform and H_2O were added to the suspension and the incubation was continued under vigorous agitation for 30 min. The phase separation was achieved by centrifugation in an Eppendorf tabletop centrifuge at 13 000 rpm for 30 min. The upper H_2O/methanol-containing phase (450 µl) was transferred in an Eppendorf tube and dried at 60°C overnight. The pellet was resuspended in 500 µl H_2O and stored at -20°C.

6.5.2. Precolumn derivatisation with FMOC

To quantitate the glutamate content by HPLC analysis, the extracted fractions were modified with 9-fluorenyl-methoxicarbonylchlorid (FMOC, Grom, Applications-Service). For this purpose, a slightly modified precolumn derivatisation method with FMOC and 1-aminoadamantan (ADAM, Grom) was applied as follows. 40 µl sodimborat buffer (0.5 M, pH 7.7) with 50 µM glycine as internal standard were mixed with 40 µl of the sample or the respective L-glutamate standard (ranging from 10 to 300 µM) and 80 µl FMOC were then added. The samples were incubated for 45 sec with vigorous shaking on an Eppendorf Thermomixer and 100 µl ADAM solution [40 mM ADAM in aceton/borat buffer, 1:2 (v/v)] were applied. After further shaking for 45 sec, 140 µl of buffer A [20% acetonitril, 0.5% tetrahydrofuran in 50 mM sodium acetate buffer (pH 4.6)] were added and the derivatisated samples were subjected to HPLC analysis.

6.5.3. HPLC analysis

To determine the glutamate content in the *B. subtilis* cells, the samples were applied in HPLC analyser (Sykam, Gilching). For the collection of the data the 5 L-glutamate standards were distributed among the samples. Subsequently 10 µl of the fluorescence-labelled amino acids were analyzed by using a precolumn (120 ODS- 4HE, 10 x 4 mm, 5 µM beads), a main column (120 ODS- 4HE, 74 x 4 mm, 3 µM beads) and fluorescence detector at an excitation wavelength of 254 nm and an emission wavelength of 315 nm. Both columns derived from Grom (Rottenburg-Hailfingen). The separation of the labelled amino acids was performed by a flow rate of 0.75 ml/min, 45°C and gradient elution with buffer A and buffer B [80% acetonitril in 50 mM sodium acetate buffer (pH 4.6)] as solvents of the system (Tab. 10).

Tab. 10. Gradient elution for amino acid separation

Time (min)	Buffer A (%)	Buffer B (%)
0	100	0
15	91	9
25	70	30
27	0	100
32	0	100
34.5	100	0
39	100	0

6.6. Determination of TreA activity

The enzymatic activity of the TreA can be easily monitored and was used for exploring promoter regions of different genes. For this purpose certain promoter regions were fused to the coding sequence of the reporter *treA* gene and the corresponding promoters were then analyzed under different conditions. The *treA* gene product in *B. subtilis* codes for the intracellular phosphor-α-(1,1)-glucosidase (TreA) and hydrolases trehalose 6-phosphate into glucose and glucose 6-phosphate. The enzyme is also able to cleave *p*-nitrophenyl α-D-glucopyranoside (PNPG) *in vitro* (Kennett and Sucoka, 1971; Helfert et al., 1995). The advantage of transcriptional *treA* fusions is the stability of TreA under high salt conditions in *B. subtilis in vivo*.

To analyze the expression patterns of the *treA* reporter fusions, 20 ml of *B. subtilis* cultures were grown at 37°C in water bath (220 rpm) under different osmotic conditions until they reached OD_{578} of approximately 1.0. The exact optical density was measured, aliquots of 1.6 ml were taken and the cells were harvested by centrifugation in an Eppendorf tabletop centrifuge at 13 000 rpm. The pellets were immediately frozen in liquid nitrogen and stored at -20°C for further use.

The TreA assay was performed as follows. The cell pellets were thawed and resuspended in 500 µl Z-buffer and 1 mg/ml lysozyme. To break down the cell wall, the suspension was incubated 5-10 min at 37°C and cell debris were centrifuged for 10 min in an Eppendorf tabletop centrifuge at 13 000 rpm. 400 µl of the supernatant were transferred in a new tube and mixed with 400 µl Z buffer. The reaction was started by addition of 200 µl PNPG (4 mg/ml in potassium phosphate buffer, pH 7.5) and incubation in water bath at 28°C. When a slight yellow colour was detected, the reaction was stopped by addition of 500 µl Na_2CO_3. In all experiment the exact time of the beginning and the end of the reaction was noted down. Absorption of the samples was measured immediately at wavelength of 420 nm and the specific activity of the phosphoglucosidase was estimated according to the formula below:

$$A_{TreA} = 1500 * E_{420} / (OD_{578} * V * \Delta t * 0.8)$$

A_{TreA}: specific TreA activity (nmol * min^{-1} * mg^{-1} = Miller unit per mg protein)

E_{420}: absorption of the cleaved *o*-nitrophenol

OD_{578}: optical density of the harvested bacterial culture

V: volume of the bacterial culture used for the assay

Δt: reaction time (from addition of PNPG to break-down via Na_2CO_3)

6.7. Amylase test

The abovementioned *treA* reporter gene fusions were integrated into *amyE* locus of *B. subtilis* after transformation by homologous recombination. The resulting strains could be easily verified for the presence of the integrants by scoring AmyE$^-$ phenotype. For this purpose, the analyzed

B. subtilis strains were plated on LB containing 1% starch. The well grown cultures were then flooded with potassium iodide solution [0.5% jod (w/v), 1% potassium iodide (w/v)] and scraped off. When *amyE* gene is present in the chromosome, the amylase would break down the starch into glucose. At AmyE$^-$ phenotype a dark violet colour of the jod-starch complexes would be seen. Alternatively, a bright halo would be formed in the presence of the amylase.

6.8. Protease assay

Secreted proteases were monitored using LB plates supplemented with 2% (w/v) skimmed milk. *B. subtilis* cultures to be tested were grown to mid-late exponential phase in LB from a freshly streaked LB plate and subsequently diluted to an OD_{578} of 0.1 in fresh LB medium. In total, 100 µl of the diluted culture was spotted onto a 2% skim milk plate and incubated at 37°C overnight. Subsequently the diameter of the halo was monitored.

IV. Results

1. High levels of DegU~P lead to the overproduction of degradative enzymes

The response regulator of the DegS-DegU two-component system has a key role in regulating several post-exponential phase processes in *B. subtilis*, including the activation and inhibition of genetic competence (Dubnau et al., 1994; Kunst et al., 1994; Ogura and Tanaka, 1996), the inhibition of flagellar-based motility (Amati et al., 2004), the activation of degradative enzyme production (Msadek et al., 1990; Dahl et al., 1992), the activation of poly-γ-glutamic acid production (Stanley and Lazazzera, 2005), and, as recently demonstrated, the activation and inhibition of complex colony architecture (Verhamme et al., 2007).

The aim of the current work was to explore the physiology of *B. subtilis* cells under hyperosmotic conditions with respect to activation of the DegS-DegU two-component system. In order to get an overview of how specific is the influence of the system on the cell physiology or on the certain target genes, three different strains were applied in the adopted experiments: *B. subtilis* wild type strain (168 or JH642, respectively), a DegU32(Hy) (Mäder et al., 2002) and [*degS degU::*aphA3] deletion mutant strain (the mutants were kind gift from Prof. M. Dahl, University of Konstanz). For more convenience, hereafter they will be referred to as hyper- and deletion mutant, respectively. In the deletion strain, both *degS* and *degU* genes were deleted from the chromosome (unpublished strain) of *B. subtilis*. In the hyper-mutant the *degU* allele carries a histidine to leucine substitution at position 12 (H12L) that is responsible for the *in vivo* effect, overproduction of degradative enzymes. However, this hyper phenotype is not due to an enhanced rate of phosphorylation of DegU but instead a decreased rate of dephosphorylation. It was demonstrated that the half-live of the DegU32 phosphate is about seven fold higher with comparison to the wild type DegU phosphate (Dahl et al., 1992).

Having in mind that: (i) the phosphorylated form of the response regulator DegU is required for the degradative enzyme synthesis (Dahl et al., 1992), and (ii) the salt stress has a strong effect on the expression of at least two genes which are known to be controlled by the DegS-DegU system, namely *sacB* (encodes levansucrase) and *aprE* (encodes alkaline protease) (Kunst and Rapoport, 1995), the abovementioned three strains were tested for their exoprotease production.

For the exoprotease assay, the strains were grown until mid-late exponential phase in LB medium and then spotted onto agar plates containing 2% skim milk. After overnight incubation at 37°C the produced halo around the colonies was monitored (Fig. 6).

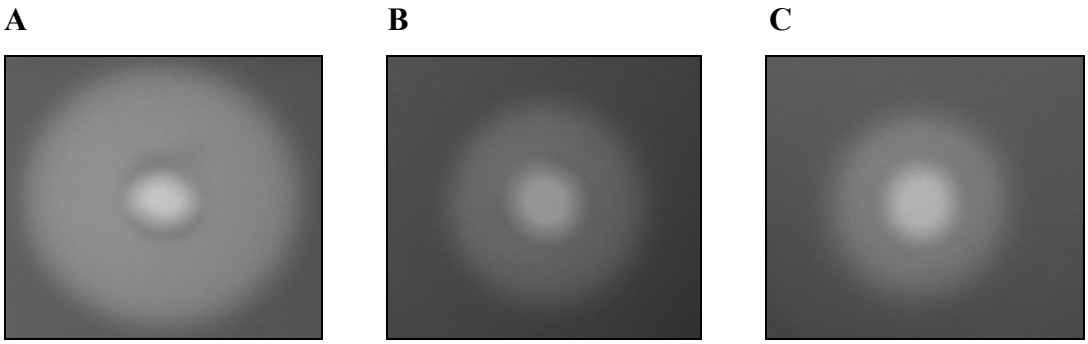


Fig. 6. High levels of DegU~P activate the production of exoproteases

Exoprotease production was monitored on 2% milk plates. The cultures were grown in LB medium until mid-late exponential phase and the synthesis of the degradative enzymes was followed for the hypper mutant MD300 (A), wild type 168 (B) and deletion mutant MD282 (C) strains.

As expected, the level of protease production in the hyper mutant strain exceeded that observed for the wild type or the deletion mutant. However, the level of the exoproteases in the deletion mutant although less was not completely abolished compared with the wild type strain. This indicates that the DegS-DegU system is not solely responsible for the production of degradative enzymes. It was shown that the exoprotease production is also positively regulated by *spoOA* (Ogura et al., 1994).

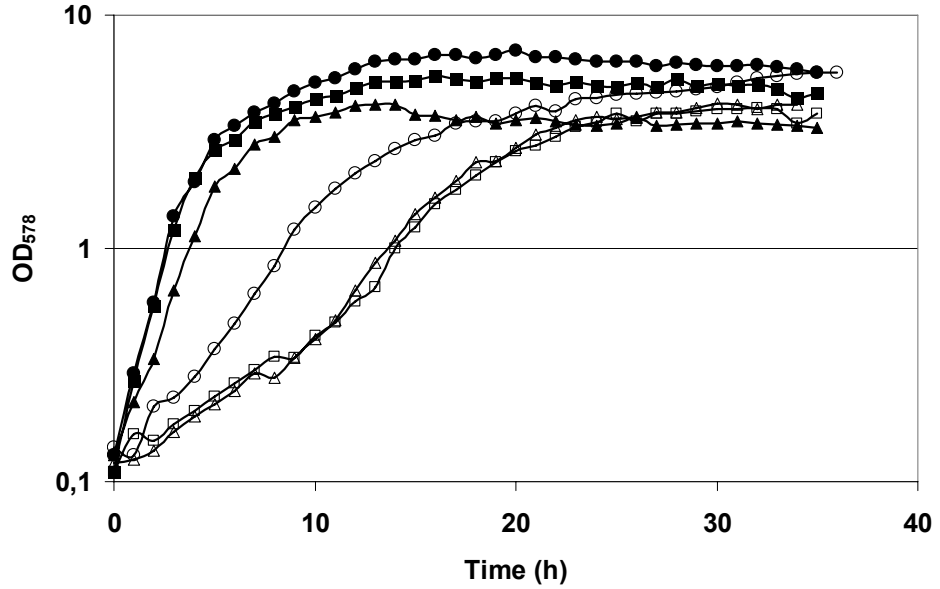
2. The *degSU* deletion mutant shows no phenotype concerning growth of *B. subtilis* in high salt conditions

To elucidate the influence of the DegS-DegU two-component system on the physiology of *B. subtilis*, the three strains mentioned above were subjected to high salt concentration and their growth curves were monitored and compared with that of the cultures not challenged with high salt (Fig. 7A). For this purpose the cultures were pregrown in 20 ml Helmann medium. Besides, each strain was cultivated in two parallel precultures – with 0M and with 0.4M NaCI medium respectively. Upon reaching the mid-exponential growth phase (OD_{578} of between 1 and 2), they were used for inoculation of 100 ml Helmann medium and 100 ml of the same medium supplemented with 1.2M NaCI. For the inoculation of the high salt medium were used the preadapted cultures and the medium was maintained so, that after inoculation to reach the desired osmolarity. The cultures were inoculated with starting OD_{578} of 1.0 and cultivated aerobically at 37°C in water bath with vigorous shaking (220 rpm). The growth was then monitored spectrophotometrically for the next 35 hours. The growth curves of the three strains did not lead to any significant differences when cultivated in a non-salt medium. They

have relatively close growth rates and reach stationary phase in about 15 hours. On the contrary, when the osmolarity of the medium was increased by the addition of 1.2M NaCI, the hyper mutant showed significantly better growth rate in comparison with the wild type or the deletion mutant, respectively. On the other hand, the growth curve of the deletion mutant did not differ from that of the wild type and both strains behave in the same way in the high salt medium. The doubling time of the wild type JH642 and the deletion mutant THB282 is 3h and 3.2 h, respectively, while that of the THB300 strain is 2.35 h. The enhanced growth of the hyper mutant demonstrated that the DegU response regulator give an advantage to the cells compromised with high salt concentrations. The lack of the phenotype on the other hand, when the genes of the system are not present in the cell, shows that *B. subtilis* is able to overcome the salt stress by other meanings.

To confirm these results, the same experiment was performed with isogenic strains, namely PB168 (*B. subtilis* wild type 168), PB5094 [*trpC2 ΔdegSU*(Kan^r)] and PB5213 [*trpC2 degU32*(Hy)]. The strains were kindly provided from Prof. A. Galizzi (Department of Microbiology and Genetics, University of Pavia, Italy). The growth curves confirmed the previous data since the wild type and the deletion mutant exhibited the same phenotype in a salt and in a non-salt growth conditions (Fig. 7B).

A



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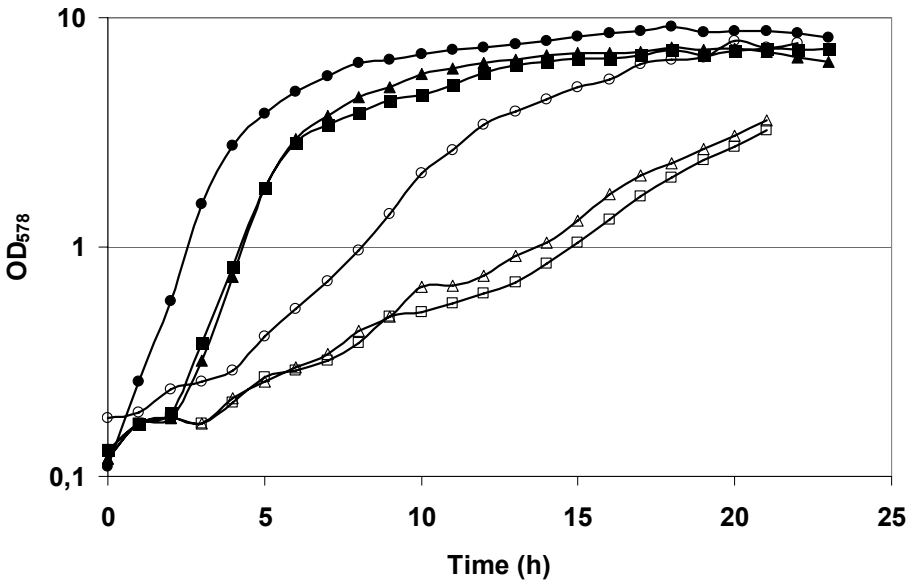


Fig. 7. The DegS-DegU two-component system influences the growth rate of *B. subtilis*

Both figures present the growth of the *B. subtilis* wild type (▲∆), hyper mutant (●○) and deletion mutant (■□) in Helmann medium without salt (▲● ■) and with addition of 1.2M NaCl (∆ ○ □), respectively. The cultures were pregrown overnight and inoculated in 0M and 1.2M NaCl media, respectively, with starting OD_{578} of 0.1. They were incubated aerobically at 37°C and vigorous agitation. The growth rate was followed until stationary phase. **A.** Growth of wild type *B. subtilis* JH642, hyper mutant THB300 and deletion mutant THB282 strains. **B.** Growth of the wild type *B. subtilis* PB168, hyper mutant PB5213 and deletion mutant PB5094 strains.

To determine more exactly at what range the high osmolarity exerts its effect, the corresponding strains were propagated in Helmann medium with gradually rising salt concentration. 20 ml of the medium were inoculated from exponentially grown overnight culture with starting OD_{578} of 0.1 and incubated aerobically at 37°C in a rotary shaker (220 rpm). The osmolarity of the medium was raised by the addition of different amount of NaCI and the resulted concentrations varied from 0 to 2 M. The cultures were then incubated for 17 hours and their optical density was monitored (Fig. 8). As it was already shown with the previous experiments, the hyper mutant exhibited a clear phenotype with respect to the high osmolarity, while the deletion mutant showed an identical growth rate as the wild type.

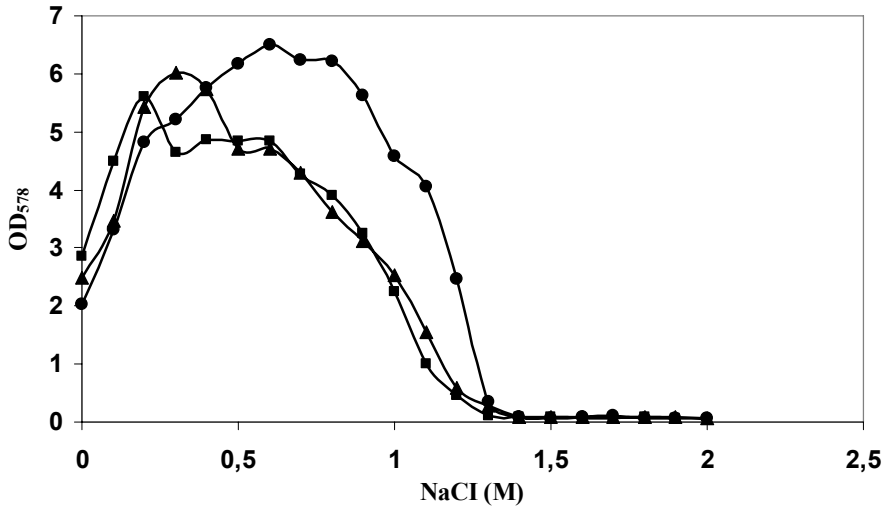


Fig. 8. Higher amounts of DegU-phosphate give an advantage of cultures grown in elevated salt concentrations

The optical density of *B subtilis* wild type JH642 (▲), hyper mutant THB300 (●) and deletion mutant THB282 (■) was measured 17 hours after inoculation in Helmann medium supplemented with NaCl concentration varying between 0.1 and 2. The cultures were inoculated with starting OD578 of 0.1 and incubated aerobically at 37°C on a rotary shaker.

Altogether, the growth of the different strains revealed that the DegS-DegU two-component system has a positive role in the adaptation of the cells to a high salt environment and that it is not the only one system involved in that process.

3. Analysis of the DegS-DegU two-component system at the transcriptional level

From the physiological data presented above it became clear that the DegS-DegU two-component system is involved in adaptation of *B. subtilis* to high osmotic conditions. To further clarify this, the role of the system was explored at the transcriptional level.

3.1. Northern blot analysis of the *degS-degU* genes

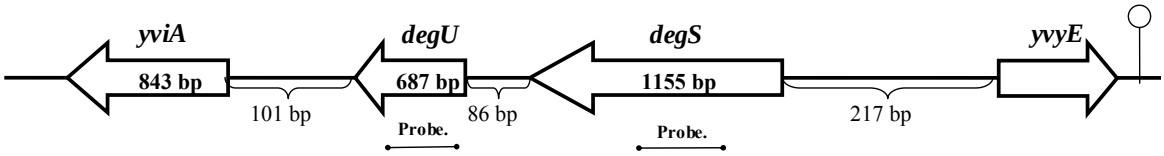
In the past, the *degS* and *degU* genes were investigated and it was suggested that they are transcribed as an operon since no obvious transcriptional terminator sequence was present between the two genes (Msadek et al., 1989). This can be also speculated from the very short intergenic region between both genes. Here, a Northern blot analysis was performed not only to confirm this assumption but also to determine the amount of the mRNA under different conditions and genetic backgrounds (Fig. 9). For this purpose, *B. subtilis* wild type (JH642 or 168), hyper mutant [DegU32(Hy)] and deletion mutant (*degS-degU*) strains were incubated at 37°C in Helmann medium alone and in Helmann medium supplemented with 1.2M NaCI,

respectively. Upon reaching an optical density of 1.0 the cells were harvested and used for total RNA isolation. About 10µg RNA were separated electrophoretically on an agarose gel, transferred to a nylon membrane and hybridized with digoxigenin-labelled anti-sense RNA probes specific to the internal region of *degS* or *degU*, respectively (Fig. 9A).

When the hybridization was carried out with degS-specific probe, there was a single band detectable with size of approximately 2.3 kb. The length of the transcript approximately corresponds to the length of the *degS* and *degU* genes together, which support the statement for the *degSU* operon (Fig. 9B). Obviously, neighbour gene *yviA*, downstream from the *degU*, is not co-transcribed because this would lead to a fragment size of more than 3 kb which is not the case. Concerning the amount of the mRNA detected in the different strains, there was no mRNA detectable in the deletion mutant which was expected. In the wild type strain, the transcript was detectable only in the salt challenged cells while in the hyper mutant it could be seen already in a non-salt conditions and it was increased in the present of 1.2M NaCI (Fig. 9B). This transcriptional profile confirmed that the higher salt concentration raises the expression of the *degS* gene. On the other hand, the higher RNA amount present in the hyper mutant hints that DegU might have an influence on the expression from the *degS* promoter. To test this hypothesis, further experiments were performed (see section 3.3.5).

To check the possibility for a separate transcription from the *degU* gene alone, another Northern blot was carried out with RNA probe specific for the latter (Fig. 9C). This led to an expression profile similar to those observed in the *degS*-Northern analysis. In the wild type strain, the transcript level was barely detectable when the cells were grown without NaCl and increased in the salt provoked cells. In the hyper mutant background, the same tendency was observed. The substantial difference here is the size of the observed transcript of about 900 bp, which indicates that the *degU* gene is transcribed alone and there is another promoter within the *degSU* operon. Besides, the amount of the RNA in the hyper mutant background was higher when compared with the corresponding bands from the wild type, which indicates that the DegU protein positively regulate its own transcription. To prove this assumption promoter fusion experiment were carried out (see section 3.3.5). As it can be seen, when the Northern analysis was performed with RNA probe against *degU* gene, there is only one band detectable on the membrane. Hence, one could argue that this might be a non-specific band since a second transcript corresponding to the whole *degSU* operon is not visible. This contradiction is due to the fact that for the visualization of the *degSU* operon transcript an overnight exposure of the membrane was necessary while the *degU* transcript could be detected within 15 min.

A



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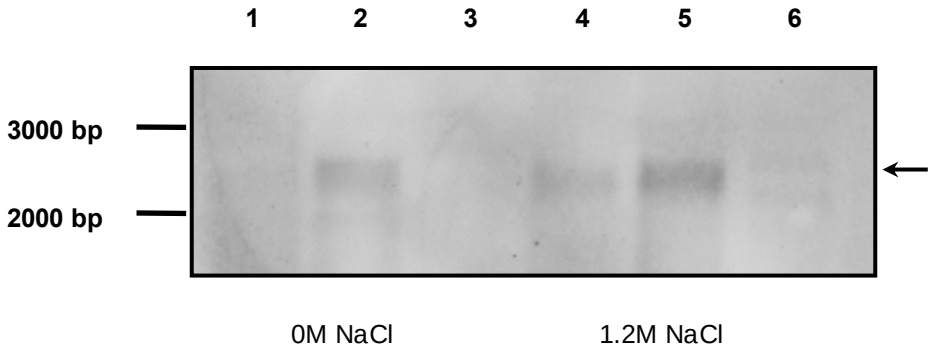

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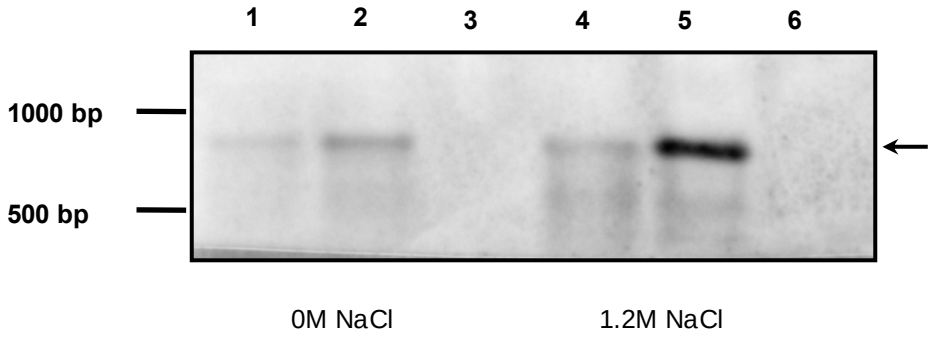

Fig. 9. Northern blot analysis of the *degSU* region

A. Genetic organisation of the *degS* and *degU* genes. Position of the RNA probes used for the hybridization studies, the size of the corresponding genes and intergenic regions are indicated. **B.** The wild type JH642 (lane 1 and 3), hyper mutant (THB300) (lane 2 and 4) and deletion mutant (THB282) (lane 3 and 6) cultures were grown in Helmann medium with 0M and 1.2M NaCl, respectively, until they reach OD_{578} of 1.0. Total RNA was isolated from the cells and the transcription profile was monitored with RNA probe specific for *degS*. **C.** The same experiment was performed by the use of *degU* specific RNA probe

Altogether the expression profiles from *degSU* operon revealed an increased transcription from both genes under high salt conditions and the presence of two separate promoters. Furthermore, the *degU* gene product seems to regulate positively the transcription from its own promoter and perhaps of the *degS* promoter as well.

3.2. Determination of the transcription initiation point of *degS* mRNA

To determine the transcription start site of the *degS* promoter with respect to the osmotic stress conditions, a primer extension analysis was performed (Fig. 10). From the Northern blot experiments it became clear that the expression from the *degS* promoter is not very high or the produced mRNA is very unstable since the band was barely detectable even after long exposure. In order to increase the amount of mRNA from the *degS* promoter, the latter was introduced in pRB373 - an *E. coli* / *B. subtilis* shuttle vector. Subsequently, *B. subtilis* wild type strain was transformed with resulted plasmid and the cultures were incubated in Helmann medium and in Helmann medium supplemented with 1.2M NaCl at 37°C and vigorous shaking (220 rpm). The exponentially grown cells (OD_{578} of between 1.0 and 1.3) were harvested and total RNA was isolated. The total RNA was then hybridized with specific primer (degSPE2) labelled at its 5' end with the infrared dye DY-781, and the hybridization product was extended with reverse transcriptase. To estimate the exact transcription start point, the *degS* promoter region was sequenced and the product was loaded next to the primer extension reaction on the gel. For the respective sequence reaction, the same labelled primer was applied. Further analysis on an LI-COR DNA sequencer revealed the transcription initiation start (Fig. 10A). The amount of the detected mRNA was greater in the preparation from the osmotic challenged cells, confirming the Northern blot analysis. Despite the different RNA preparations that were applied for the reaction, a number of minor bands were also detected. Probably these are degradation products since no other promoter could be detected.
The transcription start lies 120 bp upstream from the translation start of the *degS* (Fig. 10B). The region upstream from the transcription initiation start contains the -10 and -35 boxes that share homology with the consensus sequence of the main vegetative sigma factor (σ^A) from *B. subtilis* (Helmann, 1995). The distance between -10 and -35 region is 17 bp and a TG motif is situated directly upstream from the -10 box. Such TG motif is present in many *B. subtilis* promoter sequences and is important element for the initiation of transcription (Voskuil and Chambliss, 1998). The *degSU* promoter was already mapped in the past whereby the B. subtilis cultures were grown at 37°C (Msadek et al., 1990). The current work could confirm

the transcription start point for the *degSU* operon and in addition, it showed that the same promoter is induced when the cells are subjected to high osmotic conditions.

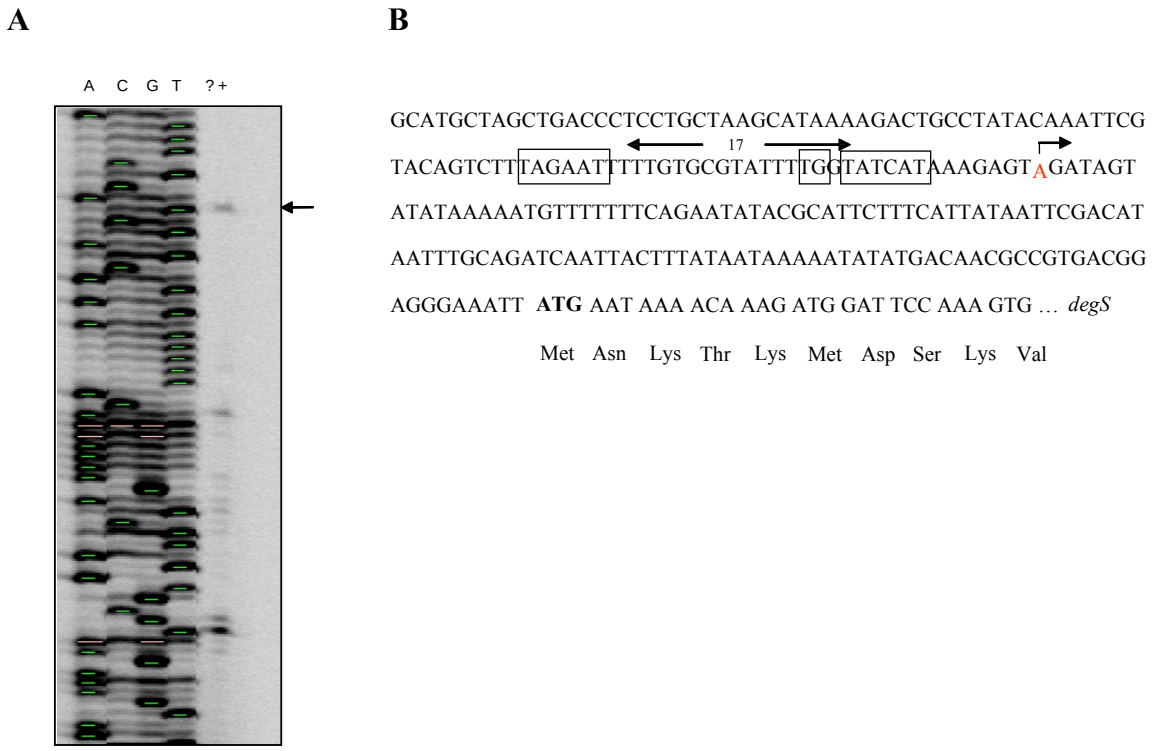

Fig. 10. Primer extension analysis of degS promoter region

A. Total RNA was isolated from *B. subtilis* 168 cultures grown in Helmann medium and in Helmann medium containing 1.2M NaCl until mid-exponential phase (OD_{578} of 1.0). The isolated total RNA was then hybridized with *degS*-specific primer labelled with the infrared dye DY-781 and the product was extended with reverse transcriptase. To estimate the size of the primer extension product, a sequence reaction of the *degS* promoter region was loaded using the same primer as a template. The reaction was analyzed on a DNA sequencer. **B.** Nucleotide sequence of the *degS* promoter region. The −10 and −35 regions of the σ^A dependent promoter and the TG motif are indicated and the transcription start point is marked with an arrow. The ATG initiation codon is showed in bold and the first following amino acid residues from the *degS* gene are written.

3.3. TreA fusion analysis of *degSU* expression in response to high osmolarity

To characterize further the osmotic regulation of the *degSU* operon, reporter gene fusions were constructed. As a reporter gene was applied the *treA* from *B. subtilis*, which encodes the salt-tolerant phospho-α(1,1)-glucosidase (TreA). The ability of the enzyme to cleave *p*-nitrophenyl α-D-glucopyranoside (PNPG) *in vitro* allows easily determination of its activity in a colorimetric reaction (Gotsche and Dahl, 1995; Schöck et al., 1996). In the first step, the corresponding promoter region of *degSU* operon was fused in frame with the coding sequence of *treA* in the vector pJMB1 (*amyE-treA-cat-amyE*). The resulted plasmid was transformed in *B. subtilis* strains, whose *treA* gene was inactivated via integration of antibiotic resistance

cassette. This led to its integration via homologous recombination as a single copy between the flanking *amyE* sequences in the chromosome of the corresponding strains. The resulting fusion strains were then used for monitoring the expression of the *degSU* promoter in response to changes in the environmental osmolarity. As a negative control served an analogous strain which carried a promoterless *treA* gene in the *amyE* locus of the chromosome. The measured enzyme activities from the control strains (TreA$^-$) were very low (data not shown) and subsequently these values were subtracted from the experimental data obtained from the fusion strains. All TreA assays described below were carried out generally in the background of *B. subtilis* JH642. Since for unknown reason there were some difficulties concerning harvesting of the cultures, the respective experiments were performed also in the *B. subtilis* 168 background which bypassed that problem. As there was no difference in the data, here just the results obtained from fusions applied in the *B. subtilis* 168 background are presented.

3.3.1. Expression of the *degSU* promoter in response to hyperosmotic shock

To explore the *degSU* regulation, a 412 bp DNA fragment carrying the promoter sequence upstream from *degS* and part of the *degS* coding sequence was cloned in front of the promoterless *treA* gene as described above. This fusion was integrated in the chromosome of *B. subtilis* strains FSB1 [JH642 *(treA::neo)*] and GSB4 [168 *(treA::neo)*] to give THB253 and THB1, respectively. As negative controls TRB0 and THB2 were applied. Here, the expression from the *degS* promoter was monitored after a sudden osmotic up-shift by means of 0.6M NaCl. To perform this, the abovementioned strains were inoculated in 200 ml Helmann medium with starting OD_{578} of 0.1 from exponentially growing precultures. They were propagated aerobically in 1000 ml Erlenmeyer flasks at 37°C and vigorous agitation (220 rpm). Upon reaching an OD_{578} of 0.4 the culture was divided in two separate Erlemmeyer flasks. Then the osmolarity of the one culture was rapidly increased by the addition NaCl in such a way that the final concentration in the medium to be 0.6M. Shortly before the osmotic up-shock and at different time points up to 3 hours after it, samples were taken out, harvested by centrifugation and subsequently used for determination of the TreA activity. In the “shocked cultures” the activity of the *degS* promoter raises from 7 to 20 units per mg protein whereas in non-shocked cells the promoter activity stays around the basic level (Fig. 11). These data correspond to results obtained from the Northern analysis and indicate that the activity of the *degS* promoter is elevated under osmotic conditions.

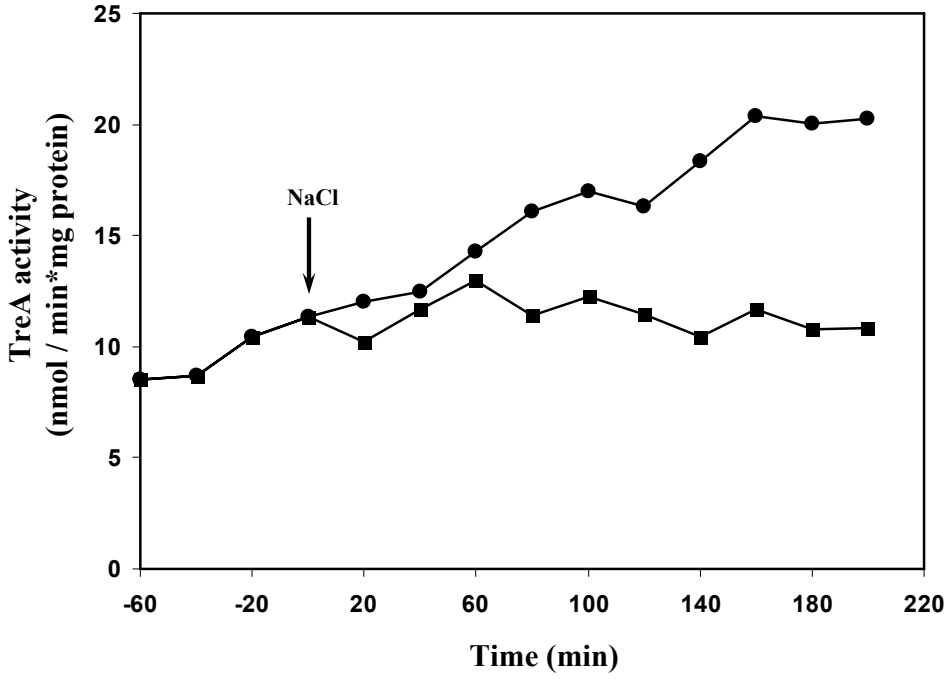

Fig. 11. TreA activity of *degS* promoter after a sudden osmotic shock

200 ml Helmann medium were inoculated with exponentially grown overnight cultures of *B. subtilis* THB1 with starting OD_{578} of 0.1 and cultivated aerobically at 37°C in a shaking water bath. Upon reaching the early exponential phase (OD_{578} of 0.4) the culture was devided and the the osmolarity of the one half was suddenly increase so that to attain a final concentration of 0.4M NaCl. Shortly before and up to 3 hours after the shock, samples were taken and the activity of the promoter was followed in a TreA assay. The presented data were obtained from three independent measurements and confirmed also for strain THB253.

3.3.2. Expression of the *degSU* promoter is dependent on the degree of the osmotic stress

It was demonstrated that the promoter upstream from the *degS* gene can respond to a sudden osmotic up-shock. Here, its expression was examined in cells grown over longer time periods under various salt concentrations. For this purpose, *B. subtilis* strains THB253, THB1, FSB1 and GSB4 were pregrown overnight in Helmann medium. Exponentially grown aliquots were then used to inoculate fresh 20 ml Helmann medium in 100 ml Erlenmeyer flasks with an OD_{578} of 0.1 and different osmolarities ranging from 0 to 1.2M NaCI. The cultures were incubated aerobically at 37°C in a shaking water bath (220 rpm) until mid-log phase (OD_{578} of 1.0-1.3) and harvested by centrifugation. The resulted pellets were finally used for determination of their TreA activity (Fig. 12). In both strains the expression of the *degS-treA* fusion was elevated and linearly correlated to the osmotic strength of the growth medium. Altogether the promoter showed about five time higher induction in the presence of 1.2M NaCI in the culture medium.

3.3.3. Influence of the osmoprotectant glycine betaine on *degSU* expression

The addition of the compatible solute glycine betaine to the growth medium can facilitate the effects of high salts on the osmoregulated gene expression (Lucht and Bremer, 1994; Spiegelhalter and Bremer, 1998). To assess its contributions to the expression of the promoter upstream from *degS*, the same experimental conditions, as described above in section 3.3.2., were applied and in addition, the cultures were supplemented with 1mM glycine betaine. The analysis of TreA activity from these cultures revealed that the *degS* promoter is still able to respond to the elevated levels of environmental osmolarity (Fig. 12). The presence of the osmoprotectant glycine betaine in the medium could not strongly reduce the promoter activity and the level of induction was retained in the same boundaries.

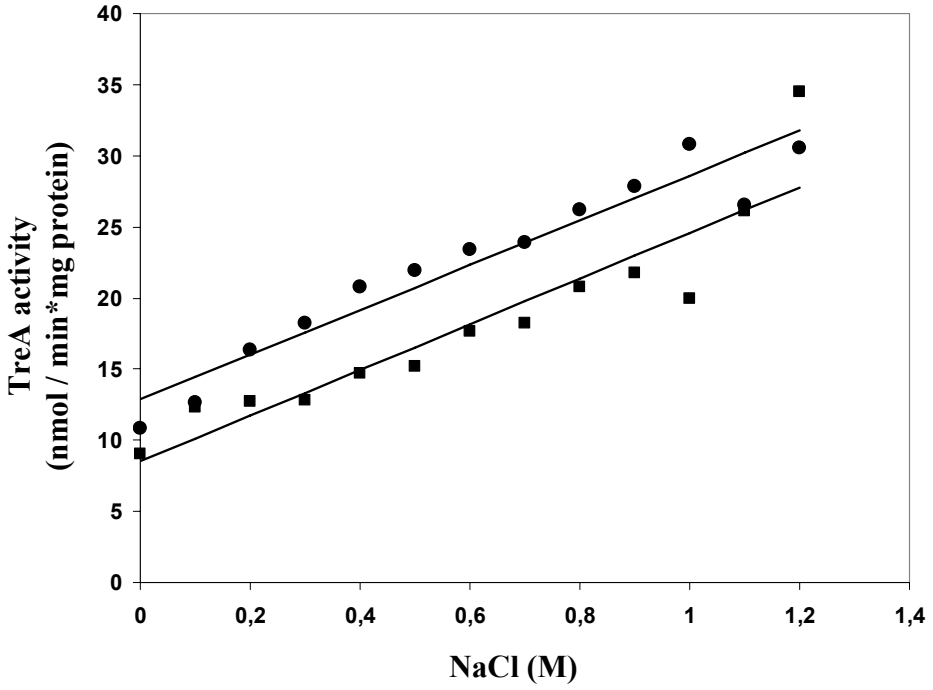

Fig. 12. Activity of the *degS* promoter is correlated with external hypertonic conditions

Precultures of *B. subtilis* strain THB1 were grown in Helmann medium overnight and subsequently inoculated in Helmann medium with ascending NaCl concentrations in the presence (■) or absence (●) of 1 mM glycine betaine, respectively. Eventually, the TreA activities of the exponentially grown cultures (OD_{578} of 1.0) were determined. Here are representatives from 3 indipendent measurements and the standart deviation did not exceed value of 0.6. This is also true for strain THB253.

3.3.4. The expression of the *degSU* promoter is subject of osmotic stimulation

The same approach was followed in order to determine whether the induction of the promoter of the *degSU* operon is observed only under hypertonic conditions as described above, or its activation is subject of an osmotic induction in general. Here, the corresponding strains were

pregrown overnight at 37°C in Helmann medium. When the cultures reached the mid-log phase they were transferred in 100 ml fresh media containing NaCI, KCI, lactose and sucrose, respectively, with osmolarity of 1100 mosmol*kg^{-1} and further incubated at 37°C in water bath shaker. Upon reaching an OD_{578} of 1.0 the cells were harvested by centrifugation and the activity of the *degSU* promoter was monitored in a TreA assay (Fig. 13). The adopted osmolarity here responds to 0.46M NaCI, 0.46M KCI, 0.68M lactose and 0.68M sucrose. It was observed that the activity of the *degSU* promoter is elevated with no consideration of the osmolyte applied in the experiment. The level of induction in the presence of NaCI or KCI was equal and corresponded to that observed in the previous experiments. However, when the environmental osmolarity was increased by the use of non-charged compounds, the promoter was induced even to a greater extent for unknown reasons. Such a phenomenon of higher activation in the presence of sucrose was also observed for the *proHJ* promoter from *B. subtilis* (Brill, 2001).

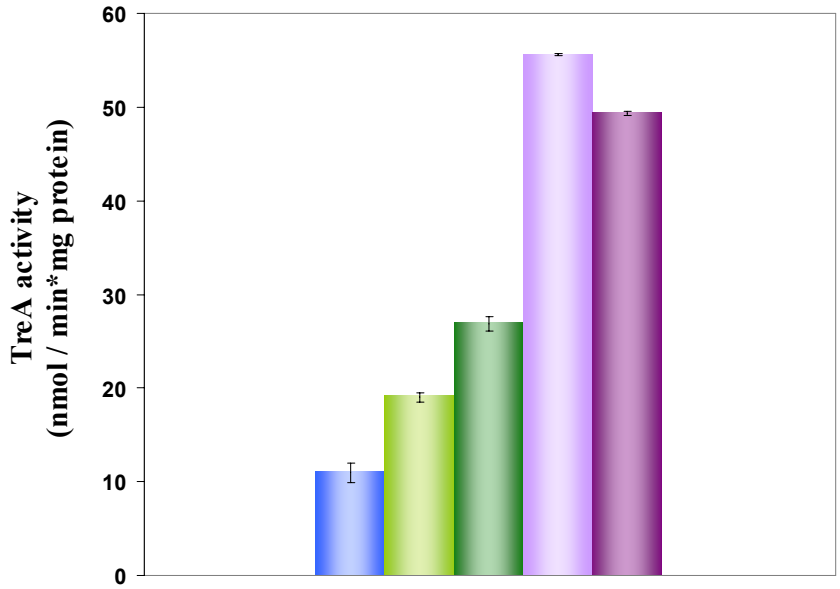

Fig. 13. The *degS* promoter is activated upon osmotic stimulation in general

Overnight cultures of strain THB1 were inoculated in Helmann medium (■) and in Helmann medium with increased osmotic strength by the addition of NaCl (■), KCl (■), sucrose (■) and lactose (■). The osmolarity values of the media were maintained to 1100 mosmol/kg. The TreA activities of exponentially grown cultures (OD_{578} of 1.0) were determined and the results from three independent experiments are plotted. The values were true for strain THB253 as well.

3.3.5. DegU-P regulates the transcription of the its own promoter

The Northern analysis of the *degS* and *degU* genes revealed that in addition to the main promoter upstream from *degS* gene, there might be another promoter within the operon which is responsible for the transcription of the *degU* only. In addition, the Northern experiments led to the suggestion that the DegU-phosphate perhaps activate the transcription from both promoters since the amount of the detected mRNA was increased in the hyper mutant strain. To confirm this hypothesis the activity of the corresponding promoters (Fig. 14A) was tested in the background of *B. subtilis* wild type, hyper mutant and deletion mutant strains, respectively. For this purpose, the following strategy was applied. The respective promoter sequences were cloned in the vector pJMB1, as it was already described, and the plasmids were introduced in the chromosome of the wild type *B. subtilis*. In the next step, the resulted strains were transformed with genomic DNA isolated from MD300 [DegU32(Hy)] or MD282 (*degS degU::aphA3*). This two-step transformation procedure was necessary because of the impaired competence of the hyper mutant strain (Msadek 1990, Dahl, 1992). For all three strains a negative controls with promoterless *treA* were constructed as well.

Initially, a 412 bp fragment containing the promoter region upstream of *degS* and part of its 5' coding region was tested according to this approach (Fig. 14B). The introduction of that region in the wild type, hyper and deletion mutant gave the strains THB1, THB31 and THB21, respectively. The corresponding cultures were grown in Helmann medium with different NaCl concentrations and subsequently tested for their TreA activities. This experiment indicated that the promoter located upstream from the *degS* gene obviously is not dependent on the amount of the DegU-phosphate since its activity was not changed regardless of the presence or absence of the DegU~P protein. Likewise, the same experiment was carried out in the presence of 1 mM glycine betaine (Fig. 14C). As it was already observed previously (section 3.3.3.) the activity of the promoter is not influenced in the presence of that osmoprotectant and the induction level is kept in the same boundaries for the three utilized strains. However, these observations contradict the Northern blot data where the mRNA level was increased in the hyper mutant. Taking into account that coping with RNA is attended with many difficulties in general, the determination of promoter activity via an enzymatic assay seems to be more reliable. For that reason, the results obtained from the promoter fusion experiment will be considered as more correct in this case.

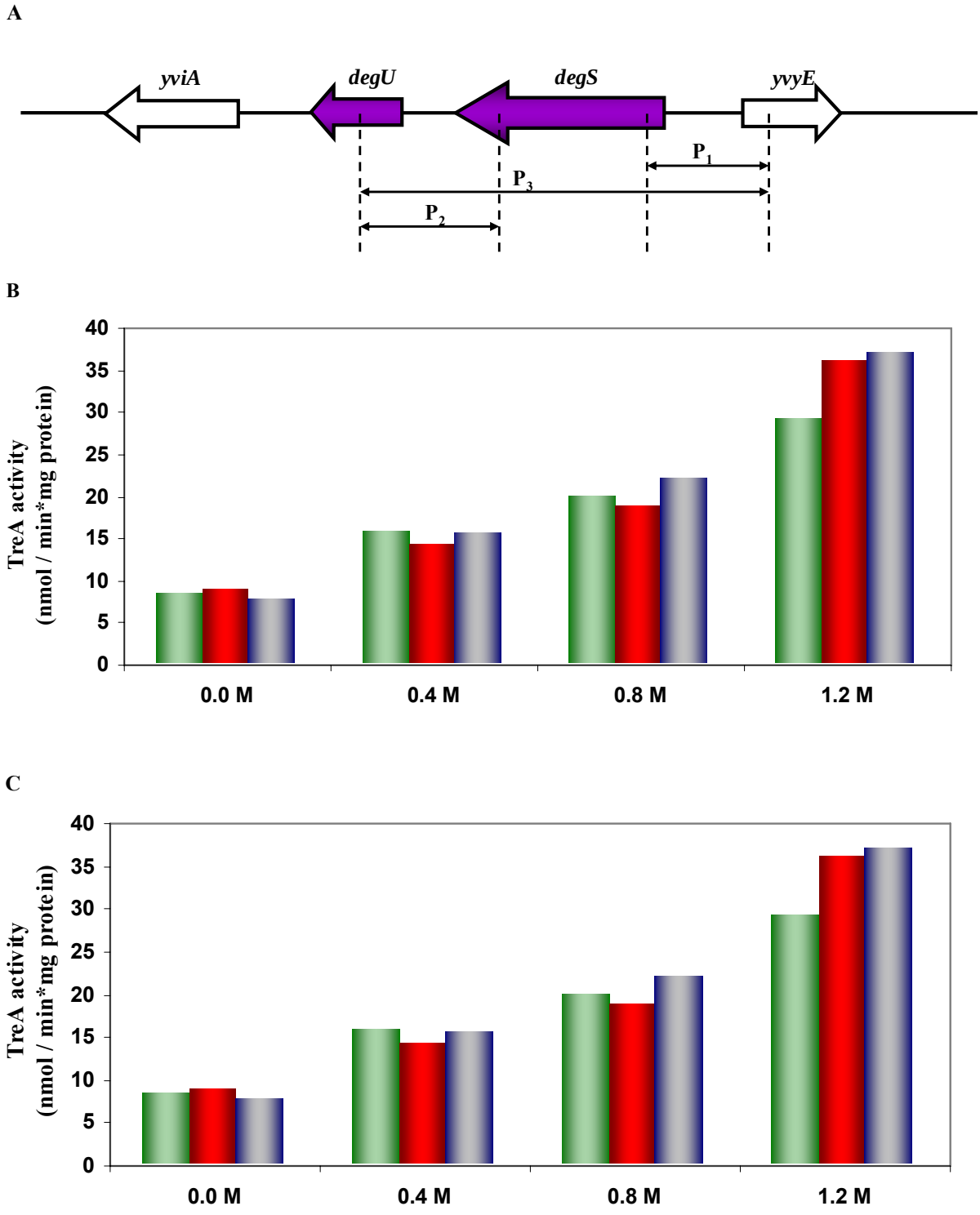

Fig. 14. The DegU-phosphate is not involved in the regulation of the *degS* promoter

A. The regions from the *degSU* operon that were used for construction of *treA*-fusions are shown with arrows. **B.** TreA assay was performed with strains THB1 (■), THB31 (■) and THB21 (■) bearing a *treA*-fusion with P_1 region. Overnight cultures of those strains were grown in Helmann medium without salt and subsequently inoculated in Helmann medium with the indicated NaCl concentrations. **C.** The same experimental procedure was followed but the cultures were incubated in media containing 1 mM glycine betaine. The data present the measurements obtained from two independently grown set of cultures.

Next, the promoter region located upstream from *degU* was explored in a similar way. For this purpose, a 454 bp fragment comprising the 3' end of *degS*, the intergenic space between *degS* and *degU*, and the 5' end of *degU* was used. The region was again cloned in vector pJMB1 and subsequently introduced in the wild type, hyper mutant and deletion mutant background using the same procedure which resulted in the strains THB417, THB317 and THB217. Here, a clear difference of the promoter activity in the corresponding genetic backgrounds was observed (Fig.15A). Namely, in the strain where the amount of DegU-phosphate was increased, the transcription from the *degU* promoter was activated to a very high level. Moreover, the observed activation is not proportional to the external osmolarity. The promoter is switched on immediately to a high level when low NaCl concentrations are present in the medium and the level of induction does not change further with the increase of the osmolarity. On the other hand, the same promoter showed moderate activation when introduced in the wild type strain along with the increases in the external osmolarity. The requirement for the DegU~P was confirmed by monitoring the activity of the promoter in the deletion mutant strain as well. In this case the TreA activities were equal with those observed in the control promoterless *treA* strain, i.e. the second promoter within the *degSU* operon functions only through DegU~P activation.

In the next experiment, the TreA activities were monitored in the presence of both promoters. In this case, a 1691 bp fragment including the promoter upstream from *degS*, the entire coding region of *degS*, the intergenic space and the 5' end of *degU* (Fig. 15B) was cloned in vector pJMB1 and the same experimental procedure was followed. Here, the activities measured in the TreA assay seem to represent some average values between those observed for both promoters separately. The overall induction level was slightly higher than that of the *degS* promoter alone, but there was no difference in the activities between the wild type and the hyper mutant background. In the deletion mutant, the TreA activities were still measurable, although lower when compared with the other two strains.

Altogether, the experiments described above confirmed (i) the presence of two promoters in the *degSU* operon and (ii) the positive feedback loop from the DegU-phosphate to the *degU* promoter.

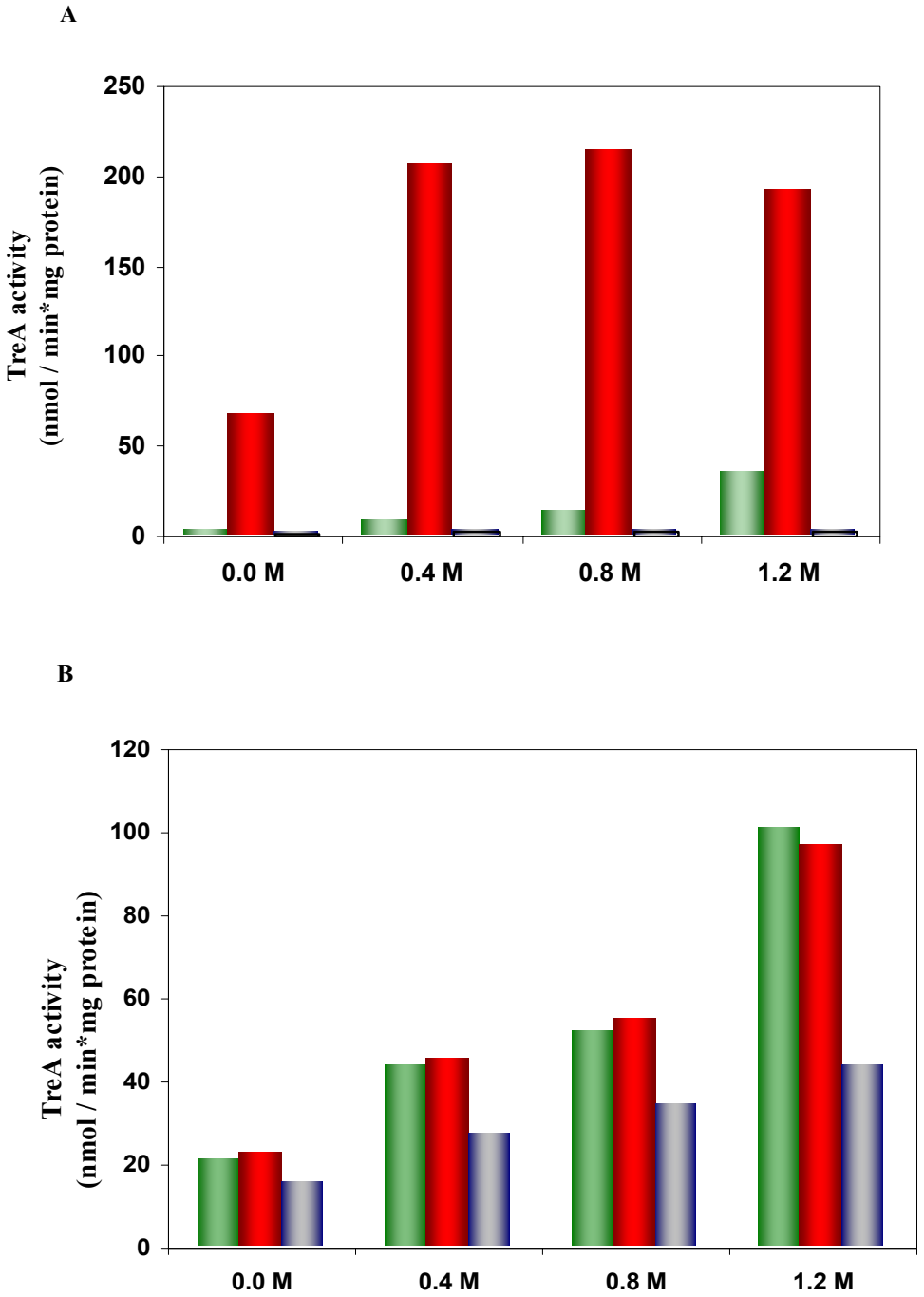

Fig. 15. The DegU-phosphate regulates positively its own promoter

A. TreA activities with the promoter region P_2 (Fig. 15A) introduced in strains THB417 (■), THB317 (■) and THB217 (■) are shown. Overnight cultures were inoculated in Helmann medium with the respective NaCl concentrations to an OD_{578} of 0.1 and incubated at 37°C and vigorous shaking. Upon reaching the OD_{578} of 1.0, they were harvested and the induction of the promoter was measured in a TreA assay. **B.** TreA activities from an analogous experiment performed with the whole *degS* gene and the 5'-region of *degU* (region P_3). The assay was carried out with strains THB416 (■), THB316 (■) and THB216 (■), respectively.

4. Analysis of the DegS-DegU two-component system at the protein level

DegS-DegU two-component system was shown to be involved in the osmotic regulation of *Bacillus subtilis* at the transcriptional level. The special feature of the system is the cytoplasmic location of its histidine kinase, whereas in the majority of known systems the kinases are membrane bound. From here arises the question how exactly the DegS sensor protein is able to sense the elevated external osmolarity. The work described in the following is focused on the more detailed characterization of the sensing properties of the DegS-DegU two-component system.

4.1. Domain organisation of DegS and DegU

DegS-DegU is a typical two-component system composed of a histidine kinase (DegS) and a cognate response regulator (DegU). The *degS* gene encodes a 385 amino acid protein with molecular mass of 45 kDa. It is composed of variable N-terminal sensing domain, extending from residues 12 to 170, and a conserved C-terminal kinase core which contains the typical dimerization and ATP-binding (kinase) domain (Fig. 16A). The dimerization domain includes the two typical conserved amino acid clusters, H-box (residues 180 to 198) and X-box (residues 225 to 245). Within the H-box is located the highly conserved histidine residue at position 189 which is the site for phosphorylation. The ATP binding domain contains the conserved N-, G1- and G2-boxes, which place the DegS in the HPK7 subfamily of histidine protein kinases (Grebe and Stock, 1999). The *degU* gene encodes a 229 amino acid protein with molecular mass of about 30 kDa. It is composed of an N-terminal receiver domain (residues 4 to 117) and a C-terminal DNA-binding domain (residues 163 to 220) which contains the typical helix-turn-helix motif (Fig. 16B). According to the sequence similarities of the output domain, the DegU belongs to the LuxR-FixJ family of response regulators.

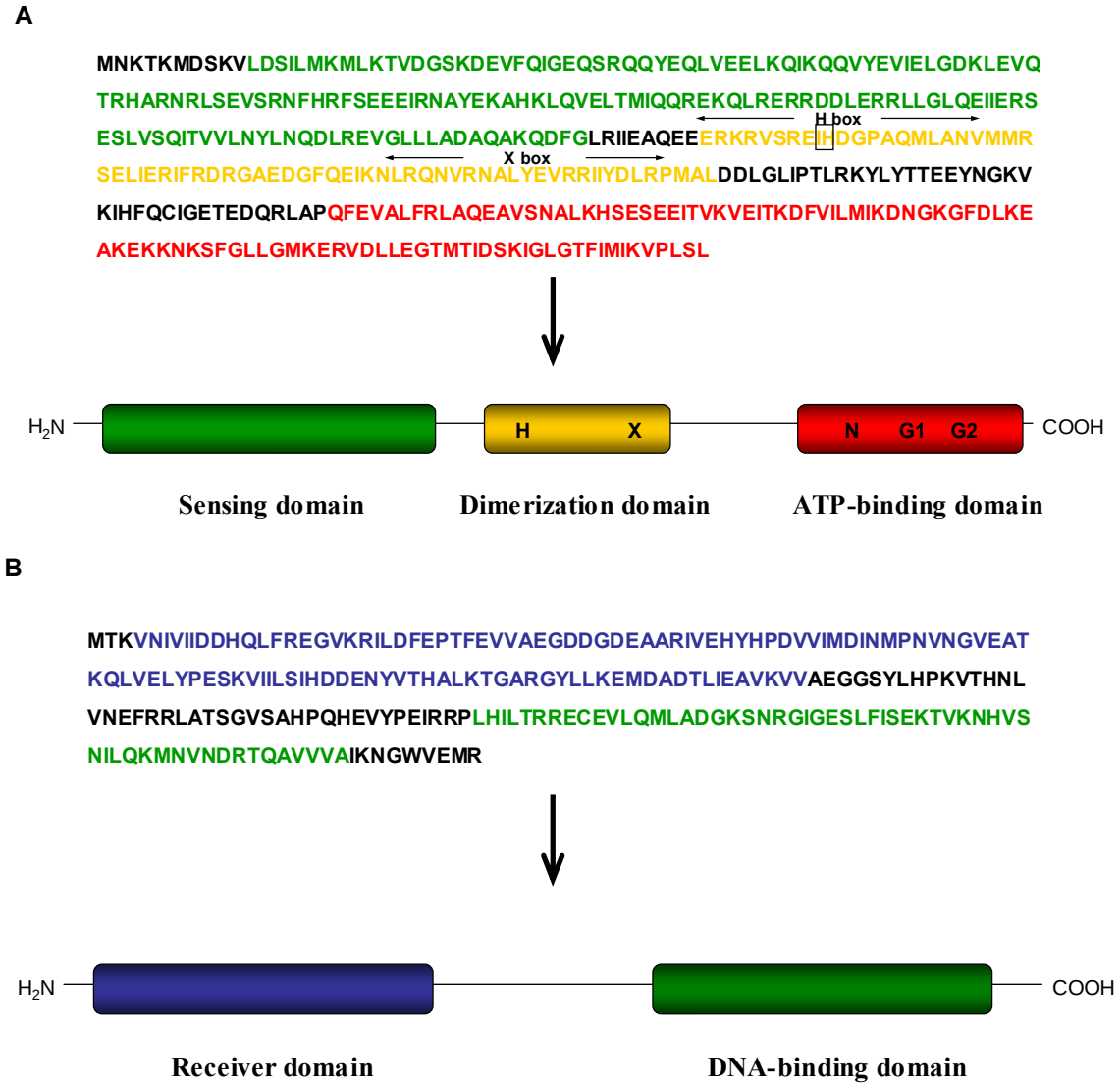

Fig. 16. Sequence-based predicted domains of DegS and DegU

The localisation of the domains as well as their corresponding amino acid residues are based on the sequence analysis from Grebe and Stock (1999) and the SMART database (http://SMART.embl-heidelberg.de) **A.** Shown are the putative sensing-, dimerization- and ATP-binding domains and the respective conserved amino acid clusters **B.** The amino acid sequences comprising the receiver and the DNA-binding domain from the DegU are indicated.

For detailed analysis of the DegS-DegU two-component system, a phosphorylation assays were performed. This required the heterologous synthesis of DegS and DegU in *E. coli*, and subsequent isolation and purification of both proteins.

4.2. Heterologous synthesis and affinity purification of DegS

For the isolation and purification of DegS, a pASK-IBA3 vector was used. In this expression plasmid, the coding region of *degS* is fused to the sequence encoding Strep-tag II at its 3' end, resulting in a C-terminal fusion with the encoded protein. The detailed construction of this

expression vector is described in section 5.5.1. (see Materials and Methods). Initially, *E. coli* BL21 cells were transformed with that plasmid and the culture was used for heterologous synthesis of the fused protein. However, after opening the cells and centrifugation of the lysate, the overprodused DegS was found in inclusion bodies in the pellet. To avoid solubilization of the protein with the means of detergents, the following strategy was performed. The plasmid encoding the DegS-Strep, was used for transformation of *E. coli* BL21 harbouring an additional plasmid pOFXtac-SL2 which encodes the *groEL-groES* genes (Castanie et. al., 1997) under an IPTG-inducible promoter. The overexpression of these chaperones should improve the yield of properly folded DegS. The resulting *E. coli* culture was then cultivated as described (see Materials and Methods, section 6.3.1.). Various induction parameters (including cultivation temperature, culture medium, inducer concentration and induction time) were examined with regard to optimal conditions for heterologous synthesis of the histidine kinase. The highest amount of soluble cytoplasmic DegS-Strep protein was achieved when the cells were cultivated in LB medium at 30°C and the protein expression was induced for about 3 hours by means of 200 µg/l AHT and 1 mM IPTG. Samples were taken before and after the induction, and the protein content was monitored via SDS-PAGE (Fig. 12). This resulted in an overproduction of a 46 kDa protein. The value matches with the expected molecular mass of the fused DegS-Strep.

For the purification of DegS, the cells were harvested about 3 hour after the induction and disrupted by three passages through a French Press. Isolation of the protein was performed via FPLC purification as already described (see Materials and Methods, section 6.3.3.). Using the given conditions it was possible to efficiently purify soluble fusion DegS protein (Fig. 17). Starting from 3 liters culture, a total yield of approximately 6 mg could be achieved.

The same procedure was followed for overexpression and purification of DegS protein with fused Strep-tag II to its N-terminus. For this purpose, a pASK-IBA5 vector was applied. However, after FPLC purification and determination of protein concentration, it was found that the amount of the isolated DegS was less when compared with the C-terminal fused protein.

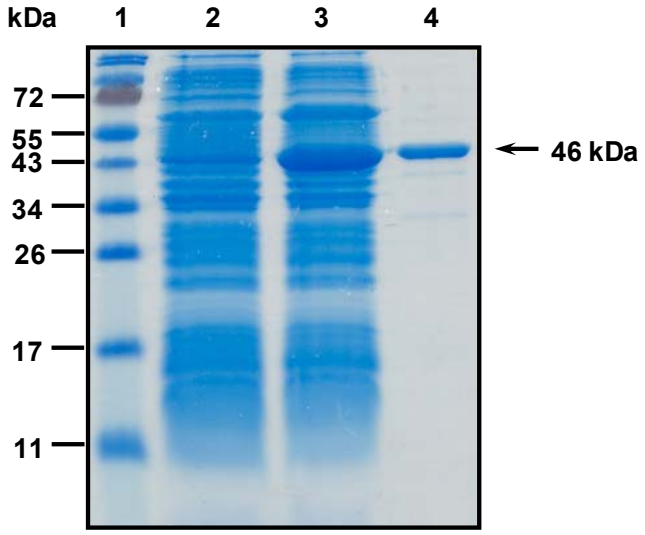

Fig. 17. Overexpression and purification of DegS

The purification procedures are described in "Materials and Methods". Samples from each step were subjected to SDS-PAGE and the proteins were stained in Coomassie Brilliant Blue. In lane 1 a prestained protein ladder is shown with the respective size in kDa on the left side. The DegS-Strep synthesis in *E. coli* BL21/ pOFXtac-SL2/ pTH3 cells before (lane2) and 3 hours after (lane3) the induction with IPTG and AHT. In lane 4 the purified DegS with a C-terminal Strep tag is present after Strep Tactin purification.

4.3. Heterologous synthesis and affinity purification of DegU

For the isolation and purification of DegU, a similar procedure was applied. The wild type *degU* gene was cloned in pASK-IBA3 vector in front of the Strep tag II coding sequence, which resulted again in a C-terminal fusion protein. The detailed construction of this expression vector is described in section 5.5.1. (see Materials and Methods). After transformation of the resulted plasmid in *E. coli* BL21 cells, the cultures were cultivated as previously described (see Materials and Methods, section 6.3.2.). Here, the use of chaperons for improvement of the protein folding was not necessary since a very high yield of soluble DegU was achieved. Nevertheless, for the adopted phosphorylation experiments several different fractions of DegU were applied. These fractions were obtained by applying a variety of induction parameters - cultivation temperature between 25 and 37°C, various AHT concentrations of 40 to 200µg/l, induction time of 15 min to 1 hour. Samples were taken before and after the induction, and the protein content was monitored via SDS-PAGE (Fig. 18). This resulted in an overproduction of a 30 kDa protein. The value matches with the expected molecular mass of the fused DegU-Strep.

For the purification of DegU, the cells were harvested and disrupted by three passages through a French Press. Isolation of the protein was performed via FPLC purification as already described (see Materials and Methods, section 6.3.3.). Depending on the applied

induction parameters, the yield of the purified protein from 3 liters culture volume varied between 3 and 12 mg.

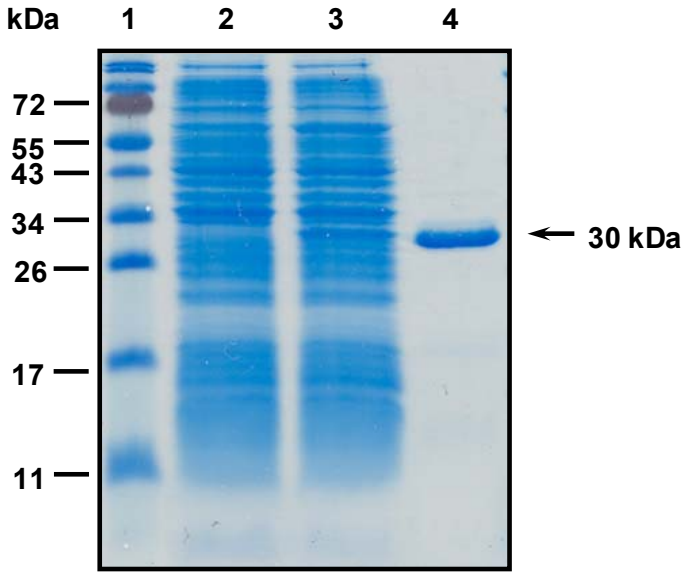

Fig. 18. Overexpression and purification of DegU

The purification procedures are described in "Materials and methods". Samples from each step were subjected to SDS-PAGE and the proteins were stained in Coomassie Brilliant Blue. In lane 1 a prestained protein ladder is shown with the respective size in kDa on the left side. The DegU-Strep synthesis in *E. coli* BL21 / pTH4 cells before (lane2) and 20 min after (lane3) the induction with AHT. In lane 4 the purified DegU with a C-terminal Strep tag is present after Strep Tactin purification.

4.4. Autophosphorylation activity of DegS

All *in vitro* phosphorylation experiments described below were, if not indicated, performed in a phosphorylation buffer (50 mM Tris-HCl pH 8.0, 10% (v/v) glycerol, 2 mM DTT, 50 mM KCl and 0.5 mM EDTA) in the presence of 20 µM [γ-^{32}]ATP and divalent cations at 37°C.

In order to determine the autophosphorylation activity of DegS after the overproduction and purification step, the sensor kinase was incubated in the presense of [γ^{32}P]ATP, subjected to SDS-PAGE and the radioactive DegS bands were quantified. The autokinase activity of DegS was determined in relation to the reaction time. Samples were taken at different time points and the time course of autophosphorylation was followed over 30 min at 37°C. It could be shown that DegS exhibits autophosphorylation activity in vitro (Fig. 19). The intensity of the phosphorylation signal strongly increased with the incubation time monitored in this assay. It also could be seen that the relation between the DegS autophosphorylation and the reaction time was almost linear.

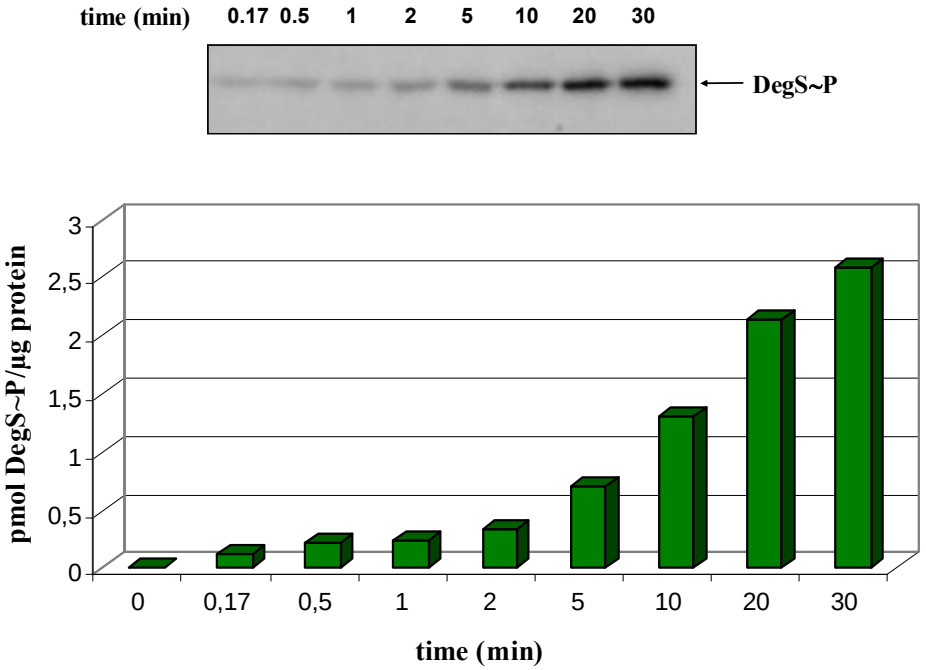

Fig. 19. Autophosphorylation activity of DegS-Strep

Shown is the time course of the autokinase activity of DegS-Strep. The protein was incubated with [γ^{32}P]ATP in a buffer containing 50 mM KCl. At the indicated time points samples were taken, separated by SDS-PAGE (upper part), and quantified with a PhosphorImager using [γ^{32}P]ATP as a standard (lower part).

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Taken together, the ability for autophosphorylation in the presence of ATP was observed with both fractions of DegS protein - containing the Strep-tag II at its C- or N-terminus, respectively. The examination of the DegS with N-terminal tag served as a control (data not shown) proving that the observed DegS activities were not influenced by the presence of the Strep-tag. Most of the subsequent experiments are performed with DegS bearing a C-terminal Strep tag.

4.5. DegS-DegU phosphotransfer

In order to shown that the whole phosphorylation cascade functions in this *in vitro* system, the phosphoryl group transfer was investigated. This was observed in a two-step reaction, as follows. Purified DegS was incubated in the presence of [γ^{32}P]ATP. After 20 min to the reaction mixture, containing the phosphorylated [γ^{32}P] DegS, a purified DegU protein was added resulting in a DegS : DegU ratio of 1 : 15. Samples were taken at indicated time points and the phosphorylation of both proteins was monitored over 15 min at 37°C. The ^{32}P was transferred from DegS to DegU, as shown by SDS-PAGE followed by autoradiography (Fig. 20). When the radioactivity found in DegS at time point T_0 was set 100%, 15 seconds after DegU addition approximately 2/3 of the radioactivity originally found in DegS was

transferred to DegU (33% of original radioactivity in DegS, 58% in DegU). Thus, only about 9% of the radiolabelled phosphoryl group was lost.

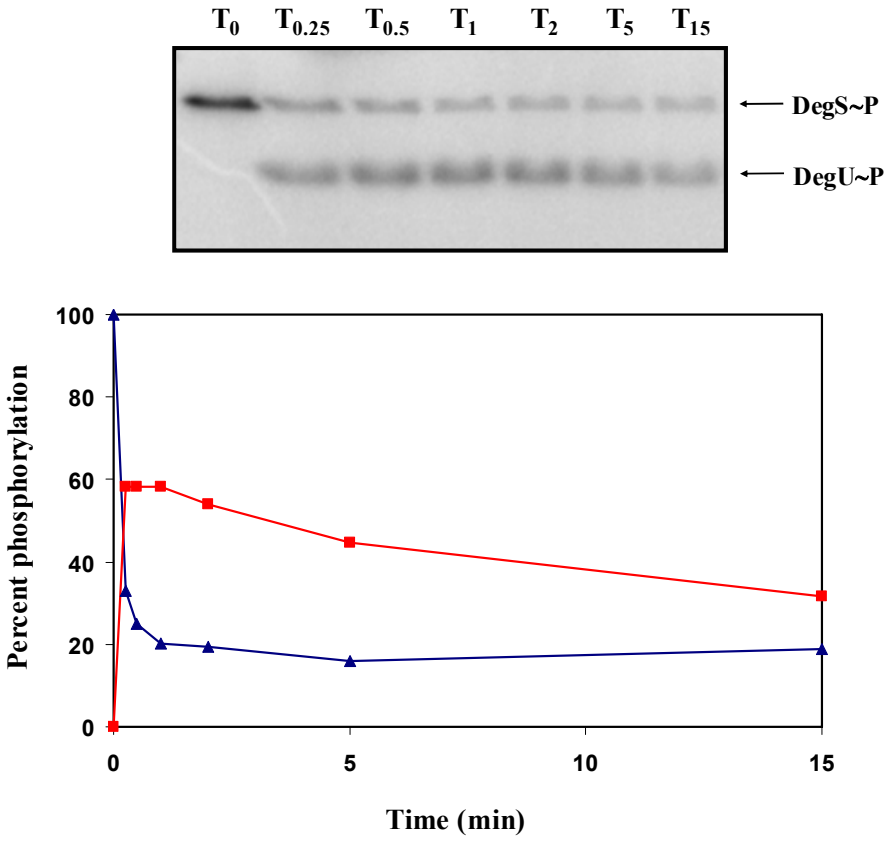

Fig. 20. Phosphoryl group transfer from DegS to DegU

DegS (1 µM) (▲) was phosphorylated under standard conditions for 20 min. At time 0 the DegU (15 µM) (■) was added to the mixture and samples were taken at the indicated time points, separated by SDS-PAGE (upper part), and quantified with a PhosphorImager (lower part). The degree of phosphorylation of DegS before the addition of DegU was set at 100%.

These results demonstrated that in this *in vitro* system DegU protein is phosphorylated in the presence of DegS kinase. Nevertheless, the response regulator appears to be quite unstable since the intensity of the radioactive band decreased very quickly after 2 minutes of incubation. It must be also noticed that the observed phosphotransfer reaction could be observed only in the presence of 300 mM Na glutamate in the corresponding phosphorylation buffer. Many different fractions of DegU protein were tested for their phosphor-accepting activities in order to improve the stability of the protein. The phosphoryl group transfer was performed with DegS and DegU proteins carrying C- or N-terminal Strep tag II, respectively, at room temperature or at 37°C and with different ratios of the proteins. Despite of all applied

conditions, the best results were observed at 37°C in the presence of 300 mM Na glutamate and C-tagged proteins.

4.6. Investigation of the influence of various solutes on the autokinase activity of DegS

The DegS-DegU two-component system was shown to be involved in sensing the high osmolarity from the environment. Since the DegS kinase appears to be a cytoplasmic protein the molecular basis of perception of a signal and its transmission to the sensor protein still remains unclear. In order to determine what might be the primary signal to which DegS responds, a series of phosphorylation assays were performed in the presence of different solutes.

4.6.1. Influence of various salts on the *in vitro* autokinase activity of DegS

Initially, the autokinase activity of the histidine kinase was tested in the presence of various monovalent salts. For this purpose, the DegS protein was incubated in the presence of 300 mM KCl, NaCl and RbCl, respectively. As a control, the same reaction was performed also in the absence of these compounds, as it was already described (section 4.4.). The histidine kinase was incubated for 20 minutes in the corresponding buffers and after indicated times samples were taken out. The intensity of the radioactive signals was monitored and the amount of the phosphorylated DegS was calculated (Fig. 21). Interestingliy, it was found the autokinase activity of DegS was not stimulated by these compounds but on the contrary, it was even inhibited to a certain extent. It seems also that the chloride anion is responsible for this inhibitory effect.

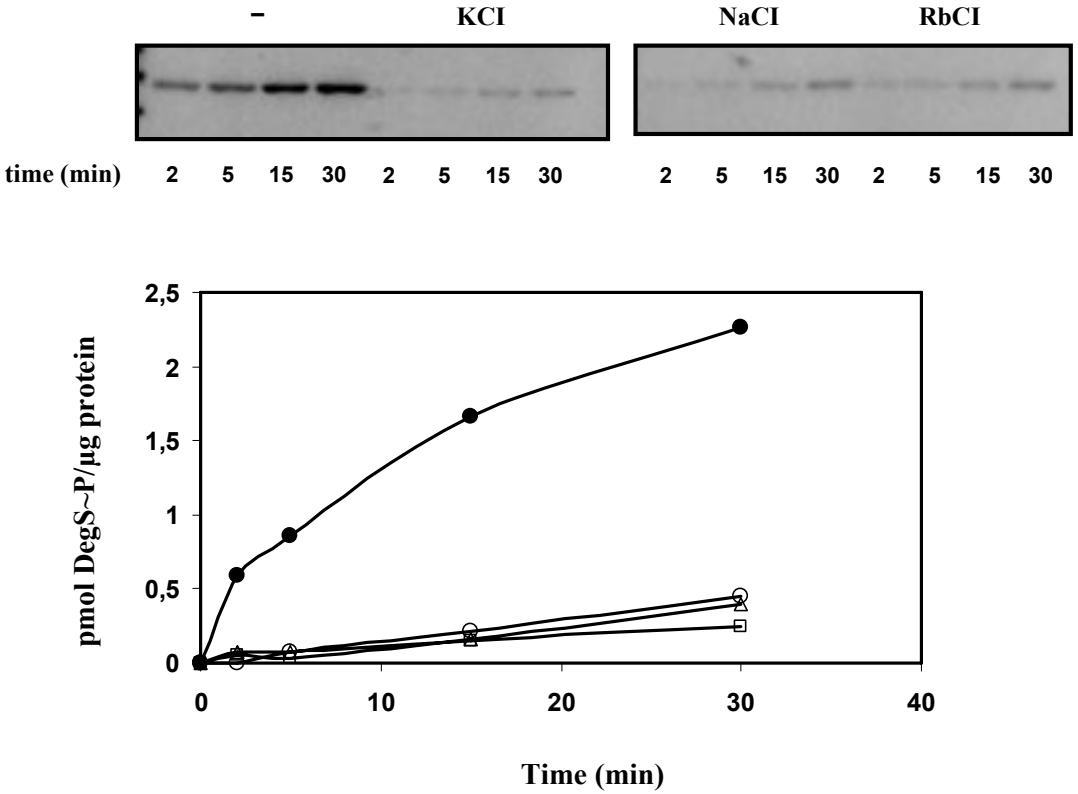

Fig. 21. Influence of various salts on the autokinase activity of DegS

DegS was incubated in phosphorylation buffer in the absence (●) or presence of 300 mM NaCl (○), KCl (□) and RbCl (Δ). Samples were taken at indicated time points, analyzed as phosphorimages (upper part) and quantified with a PhosphorImager using [γ^{32}P]ATP as a standard (lower part). The data are examples of findings from two independent experiments.

4.6.2. Influence of various solutes on the *in vitro* autokinase activity of DegS with respect to the increased osmolarity

In order to investigate if the *in vitro* autophosphorylation of DegS would be influenced from the overall increase in the osmolarity or from a sertain individual stimulus, the autokinase activity was monitored in the presence of different substrates. The DegS protein was incubated in different buffers containing proline, glycine betaine, glucose, Na glutamate, lactose and sucrose, respectively (Fig. 22). The conditions were maintained so, that the final osmolarity of the buffers to be 1700 mosmol*kg^{-1}. It was found that the glycine betaine and proline which are known to be accumulated in cells exposed to an osmotic upshift, did not influenced the DegS autokinase activity. Similarly, the utilized sugars seemed not to stimulate the autophosphorylation of the kinase. On the other hand, the addition of Na glutamate to the reaction mixture led to a significant increase in the intensity of the corresponding radioactive bands. Hence, these studies strongly indicated that the stimulation of the DegS histidine kinase is not due to an increase of the osmolarity in general but is rather a specific respond to a certain stimulus.

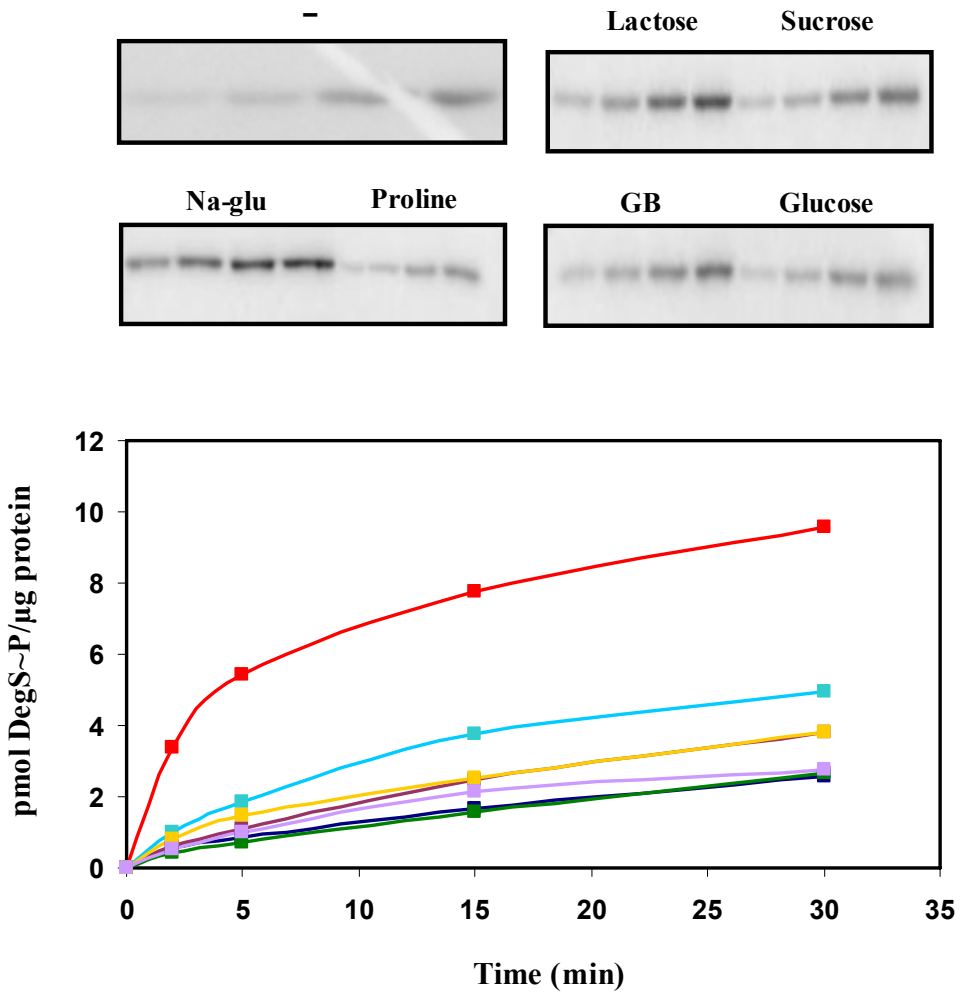

Fig. 22. Influence of various osmolytes on the autokinase activity of DegS

DegS was incubated in phosphorylation buffer in the absence (■) or presence of Na-glutamate (■), glycine betaine(■), proline(■), glucose(■), lactose (■) and sucrose (■), so that the corresponding buffers to have an equal osmotic strength. Samples were taken at indicated time points, analyzed as phosphorimages (upper part) and quantified with a PhosphorImager using [γ^{32}P]ATP as a standard (lower part). The data are examples of findings from two independent experiments.

To investigate further if the observed autokinase stimulation in the presence of Na glutamate can be really attributed to the glutamate, the autophosphorylation of the DegS was tested in buffers containing Na- and K-glutamate, respectively. The experiment was performed in an isoosmolar conditions, i.e. the glutamate concentration was kept constant and only the ratio between Na^+ and K^+ was changed gradually (Fig. 23). The resulted phosphorimages revealed that the level of the autophosphorylated DegS is retained regardless to the presented cation.

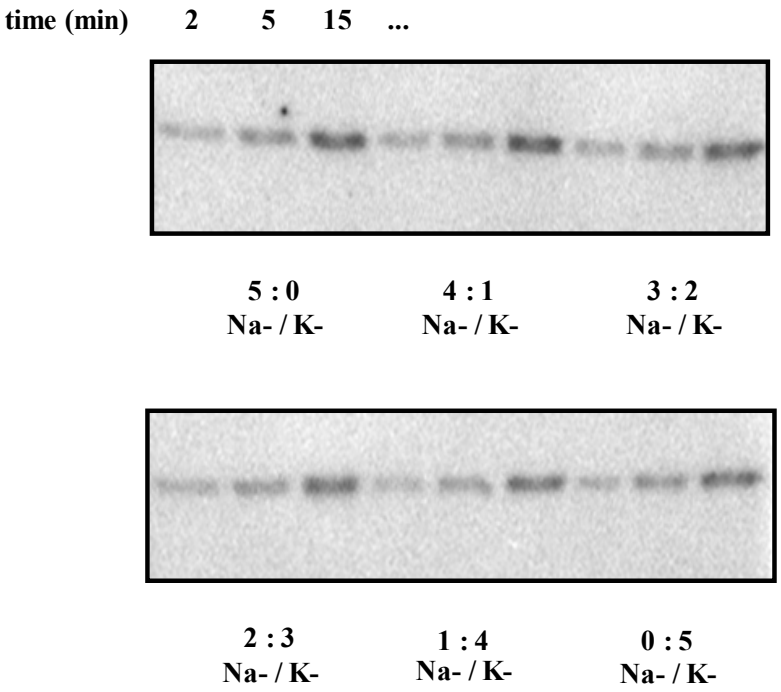

Fig. 23. Glutamate stimulates specifically the DegS autokinase activity

DegS was incubated under standard autophosphorylation conditions with buffer containing Na- and K-glutamate. The concentration of the buffer was maintained at 150 mM and only the ratio between Na^+ and K^+ was changed as indicated. Shown are the phosphorimages after SDS-PAGE separation

In the same manner, to ensure that the observed effect is related to the glutamate and not to the Na^+ or K^+, the influence of the following salts was tested: Na acetate (CH_3COONa), Na_2CO_3, Na_2SO_4, KCl, K acetate (CH_3COOK), KJ, KNO_3. This did not result in an increased level of phosphorylated DegS (Fig. 24, data shown for K-salts).

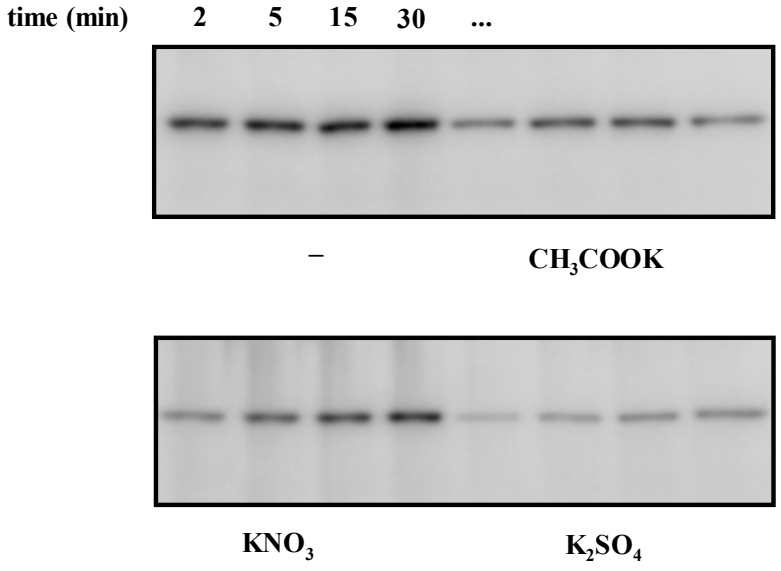

Fig. 24. K^+ does not stimulate the DegU autokinase activity

DegS was incubated with standard phosphorylation buffer in the absence (-) or in the presence of 200 mM K-acetate, KNO_3 and K_2SO_4, and samples were taken after indicated time points. Here are present the phosphorimages after SDS-PAGE separation.

4.6.3. K-glutamate influences the DegS autokinase activity in a concentration-dependent manner

In order to clarify if the DegS protein is autophosphorylated in response to glutamate content in general, or its autokinase activity is stimulated according to different glutamate concentrations, the following experiment was performed. The DegS kinase was incubated in the phosphorylation buffers with rising K-glutamate concentrations. At indicated time points samples were taken and the intensities of the radioactive bands were monitored for the whole concentration range (Fig.25). It was found that the DegS protein respond to the available glutamate in a dose-dependent manner.

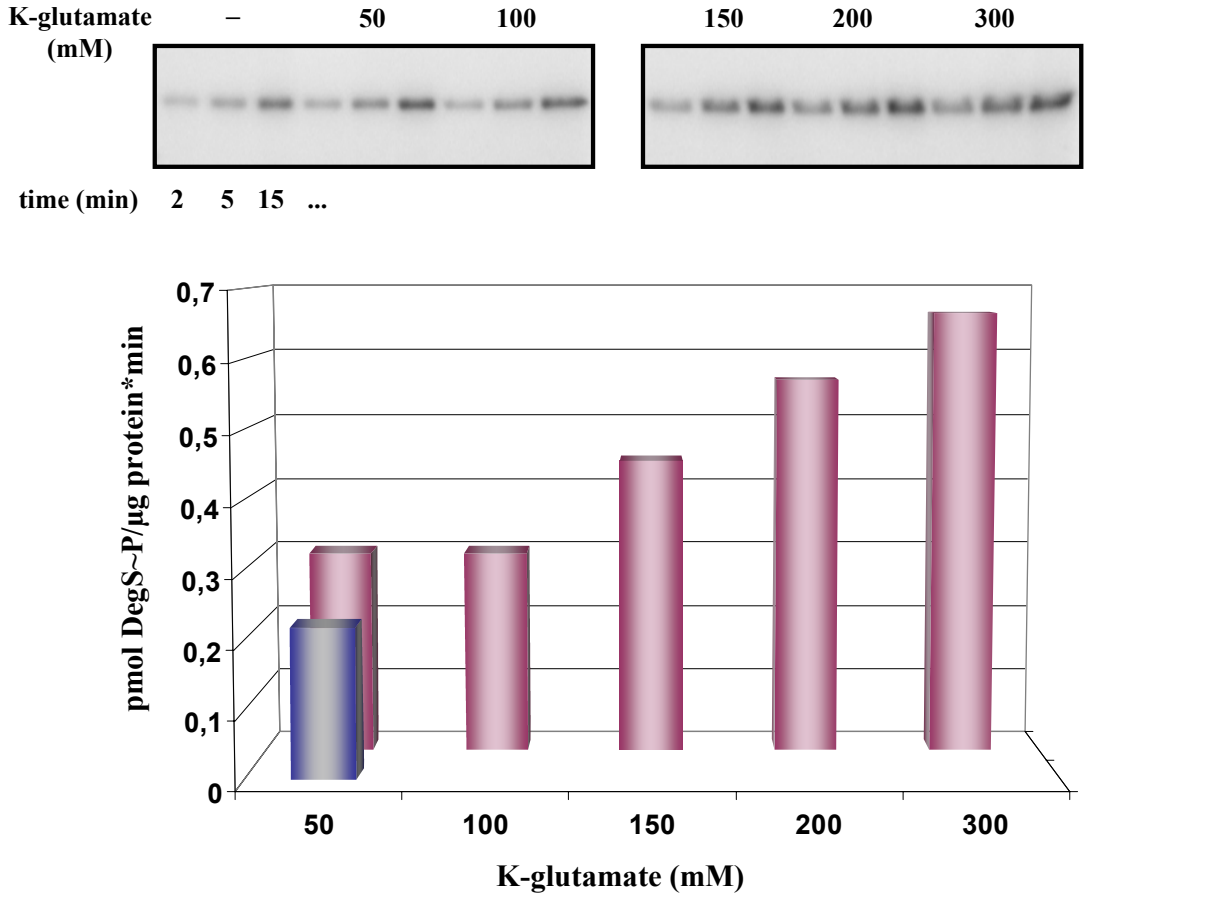

Fig. 25. K-glutamate stimulates DegS autophosphorylation in a concentration dependent manner

DegS was incubated in phosphorylation buffer in the absence (■) or presence of increasing K-glutamate concentrations (■). Samples were taken at indicated time points, analyzed as phosphorimages (upper part) and quantified with a PhosphorImager using [γ^{32}P]ATP as a standard (lower part).

Altogether, the applied *in vitro* studies with the DegS-DegU two-component system revealed that the histidine kinase does not sense the enhanced osmolarity per se but is activated specifically in the presence of glutamate.

5. Osmotically controlled glutamate accumulation

The *in vitro* assays performed with the DegS-DegU two-component system illustrated that the activity of the DegS histidine kinase is induced in the presence of higher glutamate concentrations. To investigate further if this is excusable with respect to the internal conditions of the cells exposed to high osmolarity, the glutamate content of osmotic challenged cells was quantified by HPLC analysis. For this purpose *B. subtilis* wild type strain 168 was grown in Helmann medium that is routinely supplemented with glutamate. Initially, one liter of Helmann medium was inoculated with exponentially growing precultures with starting OD_{578} of 0.1. They were propagated aerobically in 5 l Erlenmeyer flasks at 37°C and vigorous agitation (220 rpm). Upon reaching an OD_{578} of approximately 2.0 the osmolarity of the medium was increased suddenly by addition of NaCl to a final concentration of 0.4M. Shortly before the osmotic up-shock and at different time points up to 2 hours after it samples were taken out, harvested by centrifugation and subsequently used for HPLC analysis (Fig. 26).

It was already shown that the internal glutamate concentration of *B. subtilis* cells is increased after an osmotic up-shift although not to a significant level (Whatmore et al., 1990). Nevertheless, in the corresponding paper the earliest time point of monitoring the internal amino acid pool was two hours after the osmotic shock. However, the experiment performed in this study intended to follow the internal glutamate concentration of the cell immediately after the osmotic up-shift.

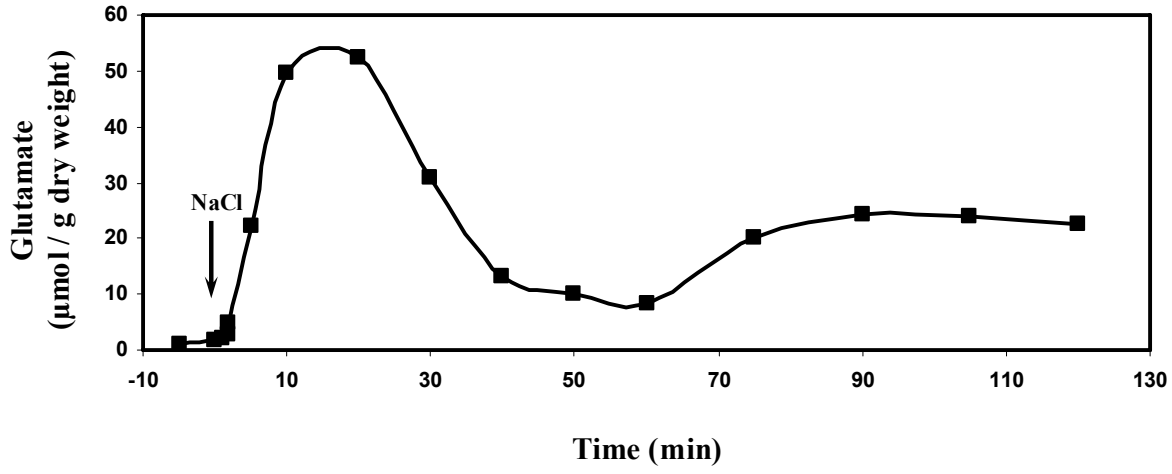

Fig. 26. Internal glutamate content in *B. subtilis* after osmotic up-shock

Cultures of *B. subtilis* 168 were grown in Helmann medium to an OD_{578} of 2.0 and the osmolarity of the medium was suddenly increased by the addition of NaCI to give a final concentration of 0.4M. Samples were taken at the indicated time points and the glutamate content was quantified by HPLC analysis. The presented data were obtained from four measurements from two independently grown cultures.

It was found that within the first 10 min after the shock, there is a sharp increase of the glutamate content which decreases after 20 min. After approximately one hour the glutamate was stabilized at a slightly elevated level (Fig. 26). These data were reproducible since those results were obtained from four parallel samples derived from two separate cultures, respectively.

6. Identification of potential DegU~P regulated targets with respect to the osmostress

Putative genes regulated from DegS-DegU two-component system were already investigated in the past (Ogura et al., 2001; Mäder et al., 2002). In this study the identification of the DegU~P regulated genes was assessed by observing two criteria: (i) the candidate genes would belong to the DegU regulon as defined from before, and (ii) the candidate genes would be upregulated in conditions of increased osmolarity in the environment. For this purpose, the following publications were utilized – Ogura et al., (2001); Mäder et al., (2002); Steil et al., (2003).

Ogura and co-workers took the strategy to identify the target genes of DegU by amplification the regulator protein in strains with disrupted sensor kinase gene. They cloned the *degU* gene on a plasmid under the control of IPTG-inducible promoter and introduced the corresponding plasmid in a *degS* null mutant. The expression level of chromosomal genes of the resulting strain was examined on a microarray. This led to the identification of 116 genes (about 2.8% of *B. subtilis* genome) which expression was affected by amplified response regulator.

Another working group (Mäder et al., 2002) characterized the DegS-DegU regulon by comparison of the gene expression patterns of the wild type *B. subtilis* and the *degU32*(Hy) mutant strain. They investigated the extracellular proteome and corresponding transcriptome by macroarray analysis in the exponential and in the stationary growth phase. Altogether they found out 32 genes which were positively regulated in the *degU32*(Hy) mutant.

In the publication of Steil et al., (2003), the cellular response of *B. subtilis* to high salinity was explored by means of microarray analysis. The transcriptional profile of salt adapted and salt-shocked cells was investigated. It turned out that 102 genes are expressed in higher levels in a high versus low-salt growth conditions.

6.1. Northern blot analysis of the DegU~P activated genes

Based on the two criteria mentioned above, the present study investigated more in detail the high salinity induced genes which belong to the DegS-DegU regulon. After comparison of the

three publications referred here, 18 genes which fulfilled those criteria were pointed out. While in the microarray analysis the expression of the global genome is explored, here the genes of interest were studied separately by the use of the Northern technique.

6.1.1. Salt-induced genes under the control of DegU~P

To determine whether certain gene is up-regulated from the DegU-phosphate, a similar procedure as for the *degSU* operon was followed. Initially, *B. subtilis* wild type, hyper mutant and deletion mutant strains were grown as described in Helmann medium and in Helmann medium supplemented with 1.2M NaCl, respectively. The exponentially grown cells (OD_{578} of 1.0) were harvested and the cell pellets were subsequently used for isolation of total RNA. Finally, the expression pattern of the genes was explored through hybridization of the isolated RNA with a single-stranded DIG-labelled RNA probe specific for the particular gene.

In Figure 27 is shown the Northern analysis of the *yddT* gene. It codes for a 228 amino acid protein with unknown function. The mRNA transcripts could be detected only from cultures grown under high salt conditions perhaps because the induction in the non-salt environment is too low. It became also clear that the *yddT* gene is up-regulated from the DegU-phosphate since the amount of the mRNA transcript was higher in the hyper mutant than that detected in the wild type strain. Additionally, the gene was expressed also in the absence of the *degS* and *degU* products although not to a very high extent. This observation implies that an additional factor is involved in the regulation of the *yddT* gene. From the size of the detected bands (approximately 1.3 kb) could be concluded that the gene is transcribed alone because the other two neighbour genes were located quite far from its coding region (Fig. 27).

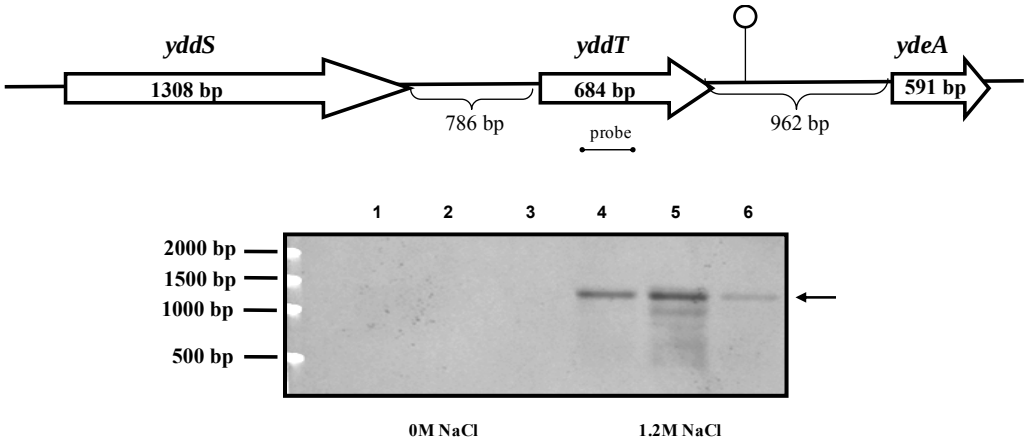

Fig. 27. Northern blot analysis of *yddT*

Shown is the genetic region of the *yddT* gene. Position of the RNA probe, the size of the corresponding genes and intergenic regions are indicated (upper part). Total RNA was isolated from the wild type JH642 (lane 1 and 3), hyper mutant (THB300) (lane 2 and 4) and deletion mutant (THB282) (lane 3 and 6) grown in Helmann medium with 0M and 1.2M NaCl, and hybridized with an *yddT*-specific probe (lower part).

Another gene that was found to have very similar transcriptional profile was *yomL* (Fig. 28). It also codes for a protein with unknown function and size of 228 amino acids. RNA transcripts could be seen also only when the cells were subjected to high salt concentration. The mRNA bands were visible in the three explored strains whereas the amount was different. Again a higher expression level was detected when higher amount of DegU-phosphate were present and on the other hand the transcription was not completely abolished in its absence. It appears that even the *yomL* gene is obviously regulated from the DegS-DegU two-component system it is not the only factor involved in that process. Concerning the size of the transcript, it was not entirely clear if this gene is transcribed alone or together with the downstream situated *yozP* (Fig. 28). The calculated size from the Northern analysis corresponds to 1.2 kb and the size of the *yozP* gene is only 327 bp which makes the estimation difficult, moreover the transcription start point of the *yomL* is not known. For that reason another Northern blot analysis was carried out by using an RNA probe specific to the *yozP* internal region. There could not be detected any signals after the hybridization (data not shown) and consequently the *yomL* gene was postulated to be transcribed alone.

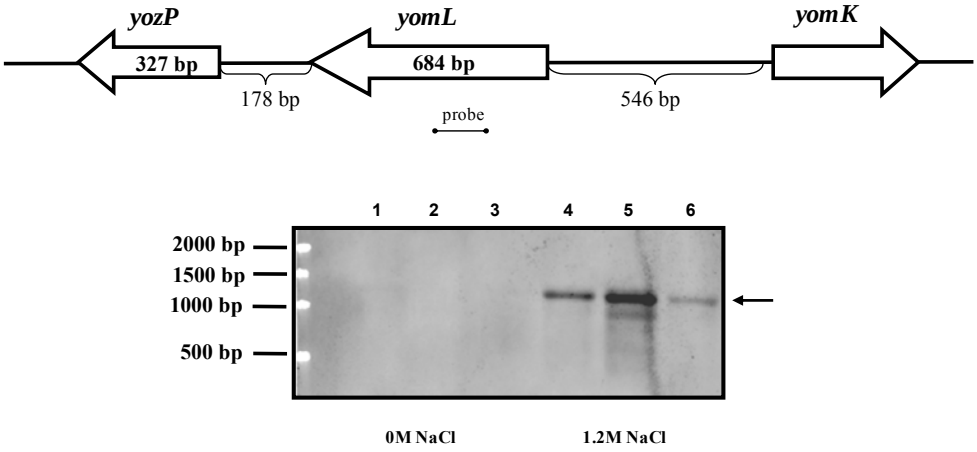

Fig. 28. Northern blot analysis of *yomL*

Shown is the genetic region of the *yomL* gene. Position of the RNA probe, the size of the corresponding genes and intergenic regions are indicated (upper part). Total RNA was isolated from the wild type JH642 (lane 1 and 3), hyper mutant (THB300) (lane 2 and 4) and deletion mutant (THB282) (lane 3 and 6) grown in Helmann medium with 0M and 1.2M NaCl, and hybridized to a *yomL*-specific probe (lower part).

Another gene which expression was followed under the adopted conditions was *yitM*. The function of the gene product is also unknown and it contains 194 amino acids. The Northern analysis revealed clearly that the *yitM* belongs to the DegS-DegU regulon since higher amount of mRNA could be detected in the hyper mutants strain in both cultivating conditions – with and without salt (Fig. 29A). At the same time, the amount of the transcript was slightly induced when the cells were subjected to high salt which indicated that the gene fulfils both

requirements mentioned earlier. Taking into account the size of the corresponding transcript of about 900 bp, it was not entirely clear if the neighbour gene *yitN* is co-transcribed (Fig. 29). In parallel to these observations, another very weak band was noticed on the same membrane with size of approximately 2.3 kb. To elucidate the transcription pattern more precisely another Northern blot was carried out with RNA probe specific to the *yitN* gene. In this case only one band with size of about 2.3 kb could be seen. Although difficult to detect, the amount of the mRNA was slightly higher in the hyper mutant in comparison with the wild type strain in the cultures grown under high salt. From size of the observed band it was evident that these genes are transcribes as an operon which includes all four genes from the region (Fig. 29B). At the same time most probably there is a second promoter within that operon upstream from the *yitM* and in the coding sequence of the *yitN* gene. In the deletion mutant background on the other hand, no transcript was detectable which shows that most likely these genes are controlled solely from DegS-DegU two-component system.

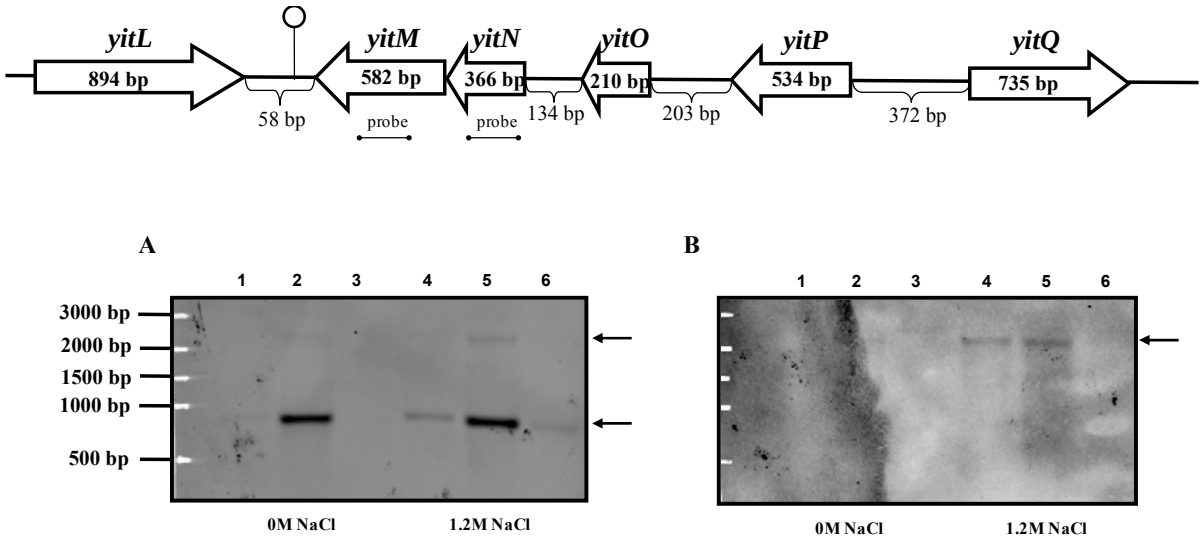

Fig. 29. Northern blot analysis of *yitMNOP* operon

Shown is the genetic region of the *yitM* gene. Position of the RNA probes, the size of the corresponding genes and intergenic regions are indicated (upper part). Total RNA was isolated from the wild type JH642 (lane 1 and 3), hyper mutant (THB300) (lane 2 and 4) and deletion mutant (THB282) (lane 3 and 6) grown in Helmann medium with 0M and 1.2M NaCl, and hybridized to a *yitM*- (A) and *yitN*-specific (B) probes (lower part).

The next gene that was determined to be a subject of salt induction and was regulated from the DegU-phosphate is *yoaJ*. The gene product constitutes a 232 amino acid protein and has an unknown function but it is similar to extracellular endoglucanase precursor. The expression pattern of the *yoaJ* revealed a higher amount of mRNA in the hyper mutant background on one hand and an additional induction when the cells were cultivated in a high salt environment (Fig. 30). It is also probable that the gene is controlled only from the DegS-

DegU system since no signal could be detected form cells with disrupted *degS* and *degU* genes. Nevertheless, one could claim that the expression pattern in the deletion mutants is ambiguous since there was also no product in the wild type strain. To clarify this, further experiments were performed (see section 6.2.). As regards the size of the detected band (about 900 bp), an operon structure with the upstream *yoaK* is excluded (Fig. 30).

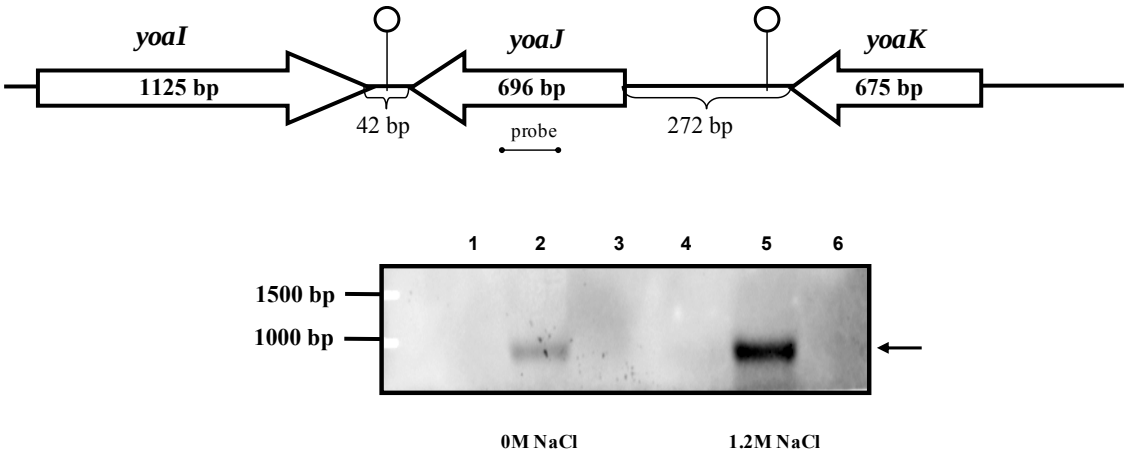

Fig. 30. Northern blot analysis of *yoaJ*

Shown is the genetic region of the *yoaJ* gene. Position of the RNA probe, the size of the corresponding genes and intergenic regions are indicated (upper part). Total RNA was isolated from the wild type JH642 (lane 1 and 3), hyper mutant (THB300) (lane 2 and 4) and deletion mutant (THB282) (lane 3 and 6) grown in Helmann medium with 0M and 1.2M NaCl, and hybridized to a *yoaJ*-specific probe (lower part).

One further gene with comparable transcription profile is *yqxI*. It encodes a small protein with 159 amino acids and unknown function as well. The Northern analysis that was carried out with specific RNA probe against *yqxI* gene revealed the presence of two bands (Fig. 31). Despite of the different RNA preparations that were applied, these two bands appeared in all experiments. Although very distinct, the upper band was considered as unspecific because its expression profile could not be attributed to the overall model exhibited by the investigated genes. The profile of the smaller band on the other hand, corresponded to a pattern where the expression level is higher in the hyper mutant strain, and even more increased in the high salt conditions. Nevertheless, no transcript was detected in the deletion mutant background from both bands which made the interpretation of the data complicated. That is why another experiment was carried out to clarify this issue (see section 6.2.). The size of the indicated transcripts was estimated to be 1 and 1.2 kb, respectively. The analysis of the corresponding gene region revealed that it is likely *yqxI* and *yqxJ* to be transcribed as an operon (Fig. 31). The upstream located *cwlA* gene is not part of that operon as it can be calculated from its size.

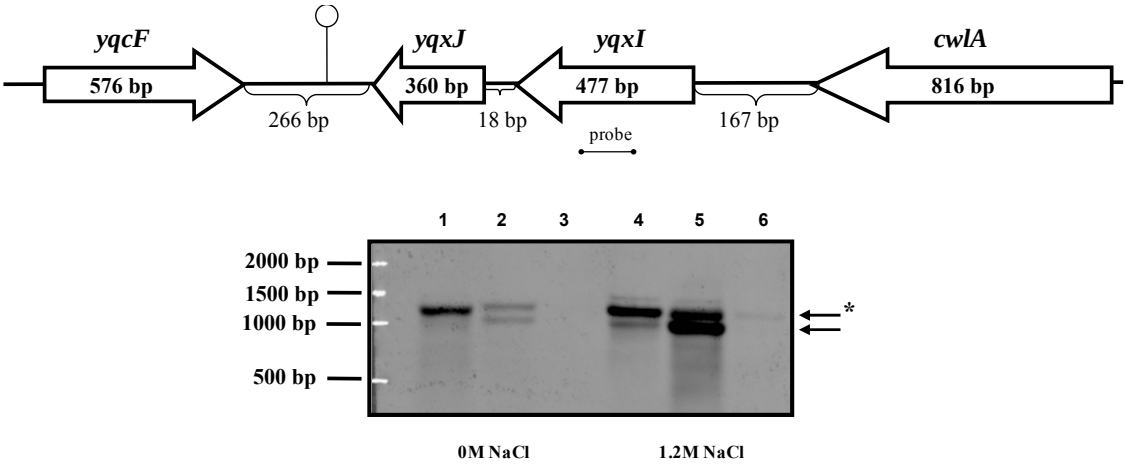

Fig. 31. Northern blot analysis of *yqxIJ* operon

Shown is the genetic region of the *yqxIJ*. Position of the RNA probe, the size of the corresponding genes and intergenic regions are indicated (upper part). Total RNA was isolated from the wild type JH642 (lane 1 and 3), hyper mutant (THB300) (lane 2 and 4) and deletion mutant (THB282) (lane 3 and 6) grown in Helmann medium with 0M and 1.2M NaCl, and hybridized to a *yqxI*-specific probe (lower part). * indicates a non specific band

6.1.2. Salt-induced genes, not regulated from the DegU~P

Following the same strategy, another group of genes was identified where the belonging to the DegS-DegU two-component system could not be demonstrated. This was the case for the *mpr* gene which encodes a 313 amino acid extracellular metalloprotease. The Northern blot analysis revealed the presence of RNA transcripts only when the cultures were incubated in high salt environment (Fig. 32A). On the other hand, the same amount of the mRNA was detected in all strains demonstrating that obviously the DegU-phosphate has no influence on its transcription. Investigation of the genetic region of *mpr* and the size of the observed mRNA transcript of about 1.4 kb implied that most probably the neighbour gene *ybfJ* is co-transcribed. Moreover the coding regions of both genes overlapped in region of 33 bp (Fig. 32). As it was already performed in case of other presumable operon structures, additional Northern analysis was carried out with specific probe against *ybfJ*. This resulted in a transcriptional pattern absolutely coincided with that for the *mpr* gene (Fig.32B). Hence, the hybridization studies here revealed that both genes formed an operon, they are salt induced and not regulated from the DegU-phosphate.

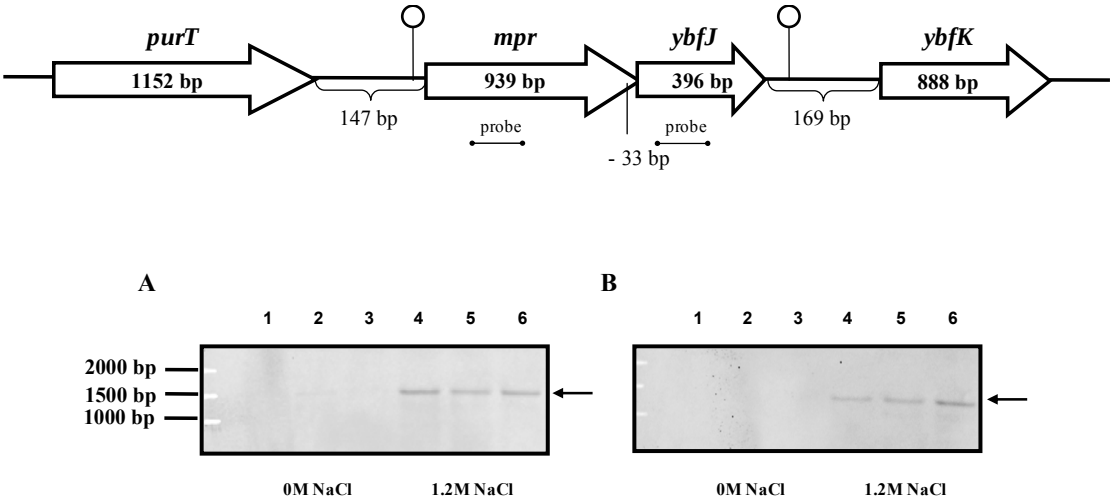

Fig. 32. Northern blot analysis of *mpr* and *ybfJ*

Shown is the genetic region of the *mpr* gene. Position of the RNA probes, the size of the corresponding genes and intergenic regions are indicated (upper part). Total RNA was isolated from the wild type JH642 (lane 1 and 3), hyper mutant (THB300) (lane 2 and 4) and deletion mutant (THB282) (lane 3 and 6) grown in Helmann medium with 0M and 1.2M NaCl, and hybridized to a *mpr*- (A) and *ybfJ*-specific (B) probes (lower part).

6.1.3. DegU~P regulated and not salt-induced genes

Another pattern that was assumed while investigating the transcriptional profile of the proposed DegS-DegU regulon, was the case where only one of the adopted criteria was observed. Such an expression showed the genes *yfjB*, *yfjC*, and *yjhA*, respectively. In order to confirm the observed model, in parallel to the Northern analysis, dot blot hybridization was performed as well (data not shown). In Figure 33 the expression of the *yjhA* gene is shown. It can be seen that the amount of the mRNA in the hyper mutant is substantially higher but the overall expression in the cultures grown in isotonic conditions prevail that of the cultures exposed to high salt concentrations. The estimated size of the detected transcript (Fig. 33) showed that the *yjhA* gene is transcribed alone.

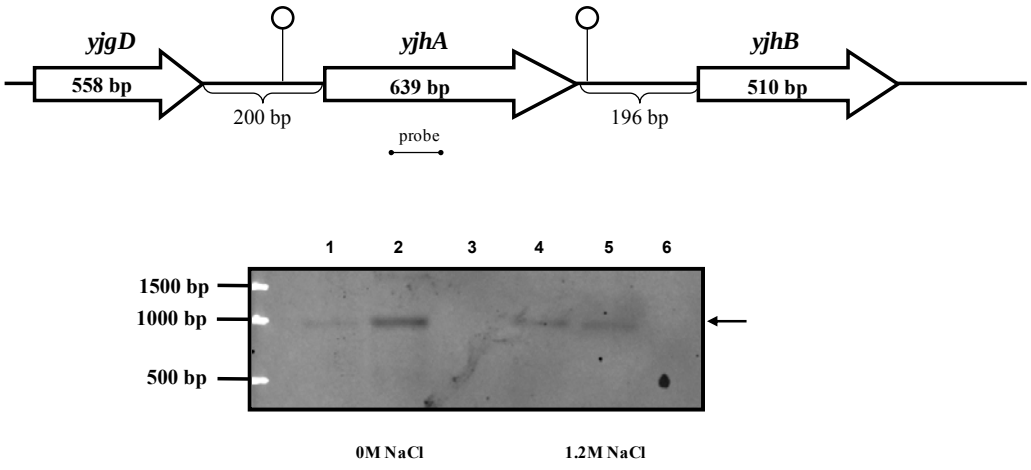

Fig. 33. Northern blot analysis of *yjhA*

Shown is the genetic region of the *yjhA* gene. Position of the RNA probe, the size of the corresponding genes and intergenic regions are indicated (upper part). Total RNA was isolated from the wild type JH642 (lane 1 and

3), hyper mutant (THB300) (lane 2 and 4) and deletion mutant (THB282) (lane 3 and 6) grown in Helmann medium with 0M and 1.2M NaCl, and hybridized to a *yjhA*-specific probe (lower part).

For the rest of the investigated genes Northern analysis did not lead to a clear expression model (data not shown) and for this reason they were not further explored. Here were assigned *yukC*, *yukE*, *ywqH* and *ywqJ*, respectively.

Altogether, analysis of the DegU~P activated genes in the presence of high salt concentration via Northern blot revealed several patterns of expression which are summarized in the table 11 below.

Table 11. Summary of the investigated genes in the presence of elevated DegU~P concentrations and high salt environment

Gene	DegU~P regulated	Salt-induced	Potential function	Potential signal sequence	Predicted TMHs
mpr	-	+	Extracellular metalloprotease	+	-
yddT	+	+	unknown	+	-
yfjA	n.d.	n.d.	unknown	+	-
yfjB	+	-	unknown	+	-
yfjC	+	-	unknown	+	-
yfjD	n.d.	n.d.	Unknown, similar to unknown proteins from *B. subtilis*	+	2
yitM	+	+	Unknown, similar to unknown proteins from *B. subtilis*	+	1
yitN	+	+	Unknown, similar to unknown proteins from *B. subtilis*	+	3
yitO	+	+	Unknown, similar to unknown proteins from *B. subtilis*	+	1
yitP	+	+	Unknown, similar to unknown proteins	+	-
yjhA	+	-	Unknown, putative lipoprotein	+	-
yoaJ	+	+	Unknown, similar to extracellular endoglucanase precursor	+	-
yomL	+	+	unknown	+	-
yqxI	+	+	unknown	+	-
yqxJ	+	+	unknown	-	-
yukC	n.d.	n.d.	Unknown, similar to unknown proteins	-	1
yukD	n.d.	n.d.	Unknown, similar to unknown proteins	-	-
yukE	n.d.	n.d.	unknown	-	-
ywqH	n.d.	n.d.	unknown	-	-
ywqI	n.d.	n.d.	Unknown, similar to unknown proteins from *B. subtilis*	-	-
ywqJ	n.d.	n.d.	Unknown, similar to unknown proteins from *B. subtilis*	-	-

n.d., for those genes the Northern analysis did not lead to any significant bands. The prediction of potential signal sequences and transmembrane helices were performed with the programs SignalP 3.0 (http:// www. cbs.dtu.dk / services/SignalP/) and TMHMM 2.0 (Transmembrane Protein Topology with a hidden Markov Model; http://www.cbs.dtu.dk/services/TMHMM-2.0)

Many of the identified genes encode proteins of unknown function which in most cases have predicted signal peptides. This is in a good agreement with one of the processes regulated by the DegS-DegU two-component system, namely the production of extracellular degradative enzymes.

6.2. Promoter-fusion assay with the candidate DegU~P activated targets

The performed Northern blot experiments could not refer all of the investigated genes to the DegU regulon activated through increased salt concentrations. Nevertheless, the transcriptional profile for 9 of the investigated genes fit to the primary requirements, i.e. they are up-regulated in the presence of higher amounts DegU~P and under increased salt concentration. Additional confirmation supporting further the role of the DegS-DegU two-component system in regulating those targets was obtained through reporter gene fusions. Here, the same strategy was followed, as for the *degS* and *degU* promoter investigations. The promoter regions of the corresponding genes were fused to the coding sequence of the *treA* gene and the activity of the promoter was measured subsequently in a TreA assay. To get information if the DegU-phosphate is really involved in activation of those promoters, the constructed fusions were introduced in the wild type, hyper mutant and deletion mutant strain, respectively.

6.2.1. Activities of the *yddT*-promoter region

A 437 bp fragment containing the promoter region of *yddT* was fused to the *treA* coding sequence on vector pJMB1 to give the plasmid pTH10. After introducing the resulted fusion in the three strains mentioned above, the cultures were grown in Helmann medium and in Helmann medium supplemented with 1.2M NaCl until mid-exponential phase. The cells were harvested and used for determination of the promoter activity (Fig. 34). When the *yddT* promoter was introduced in the hyper mutant background, its activity was clearly higher compared with that of the other strains. The pattern observed here confirmed the results from the Northern analysis also in other aspect, namely the activity of the promoter is not inhibited in the absence of the DegU regulator. Hence, *yddT* is under the control of the DegS-DegU system but for its activation is responsible also an additional unknown factor.

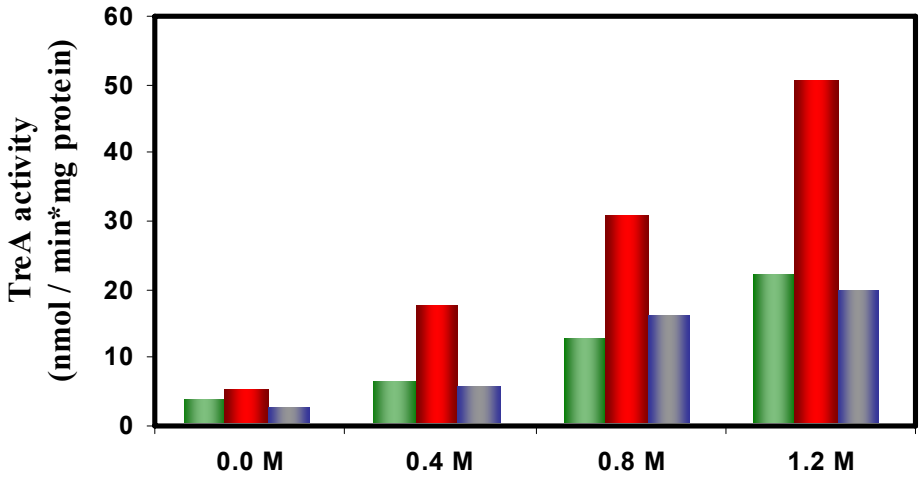

Fig. 34. TreA activities of the *yddT* '- *treA* fusion

TreA assay was performed with strains THB410 (■), THB310 (■) and THB210 (■) bearing a *treA*-fusion with the *yddT* promoter region. Overnight cultures were grown in Helmann medium without salt and subsequently inoculated in Helmann medium with the indicated NaCl concentrations. The data present the measurements obtained from two independently grown set of cultures.

6.2.2. Activities of the *yitMNOP* operon region

Initially, only the affiliation of the *yitM* gene to the DegU regulon was explored. In the course of the experiments it became clear that in addition to *yitM*, *yitN*, *yitO* and *yitP* genes are likely to be transcribed as an operon under the control of DegU~P. For this reason the whole region was fused to the *treA* coding sequence and further explored for the activity of the trehalose-6-phosphate (TreA) (Fig. 35A). Again, the highest activity was observed when the DegU~P was presented in a greater amount and by this way confirming the Northern data. In the same time, there was absolutely no activity in the absence of *degS* and *degU*. But the difference between the wild type and the hyper mutant on the other side was not substantial. This could come from the difficulties of transcription from the long mRNA and perhaps some complex secondary structures - the cloned region has a size of 1856 bp. To clarify this, an additional promoter fusion experiment was carried out where only the promoter region of first gene from the operon (*yitP*) was used. The cultures were cultivated under the same conditions as before and the performed assay supported the previous findings (Fig. 35B). The *yitP* promoter showed higher activity in the hyper mutant background and also induction upon hypertonic conditions.

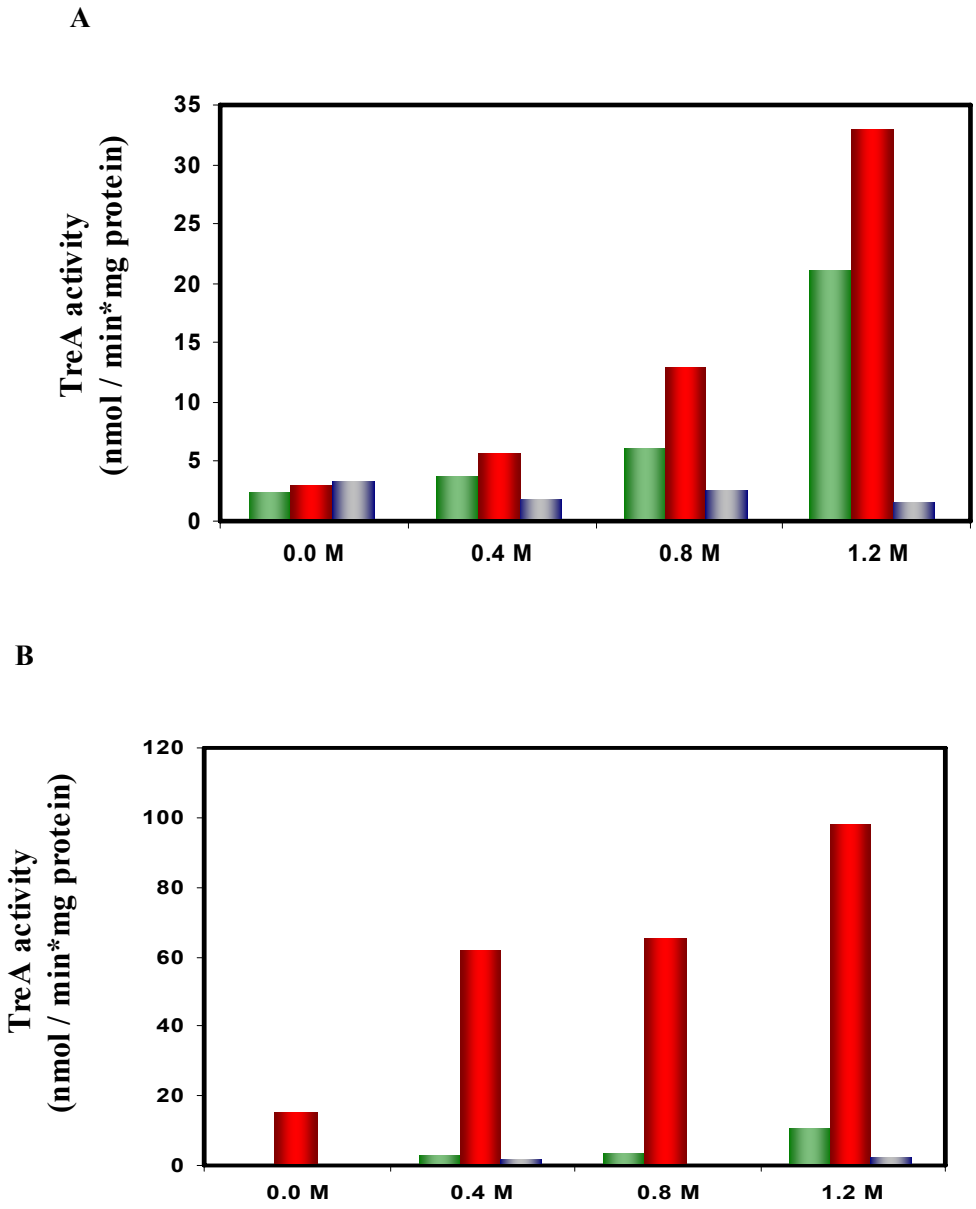

Fig. 35. TreA activities of the *yitMNOP* '- *treA* fusion

For the TreA assay overnight cultures were grown in Helmann medium without salt and subsequently inoculated in Helmann medium with the indicated NaCl concentrations. **A.** Shown are the data from strains THB411 (■), THB311 (■) and THB211 (■) bearing a *treA*-fusion with the *yitMNOP* region **B.** Shown are the data from strains THB412 (■), THB312 (■) and THB212 (■) bearing a *treA*-fusion with the *yitP* promoter region. The results were obtained from two independently grown set of cultures.

6.2.3. Activities of the *yoaJ*-promoter region

A 317 bp promoter region upstream from *yoaJ* was cloned in the vector pJMB1 and the same experimental procedure as described was performed. The TreA assay presented the same transcriptional profile (Fig. 36) as already observed in the Northern analysis. With comparison to wild type and deletion mutant, the promoter activity was switched on to a very high level in the background of the hyper mutant and hypertonic environment.

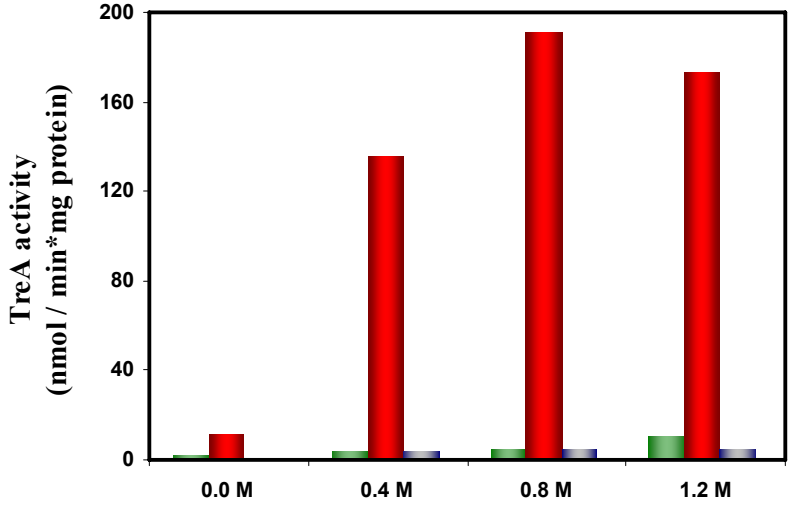

Fig. 36. TreA activities of the *yoaJ* '- *treA* fusion

TreA assay was performed with strains THB413 (■), THB313 (■) and THB213 (■) bearing a *treA*-fusion with the *yoaJ* promoter region. Overnight cultures were grown in Helmann medium without salt and subsequently inoculated in Helmann medium with the indicated NaCl concentrations. The data present the measurements obtained from two independently grown set of cultures.

6.2.4. Activities of the *yomL*-promoter region

To explore the activity of the *yomL* promoter, a 315 bp region upstream from its coding sequence was cloned in vector pJMB1 and the same experimental conditions were applied as described. The results from the TreA assay (Fig. 37) fully coincided with the previously done Northern analysis and confirmed the affiliation of the *yomL* to the DegU regulon. They also proved that an additional factor is involved in the regulation of the corresponding gene since its activity was still switched on when *degS* and *degU* were not present on the chromosome.

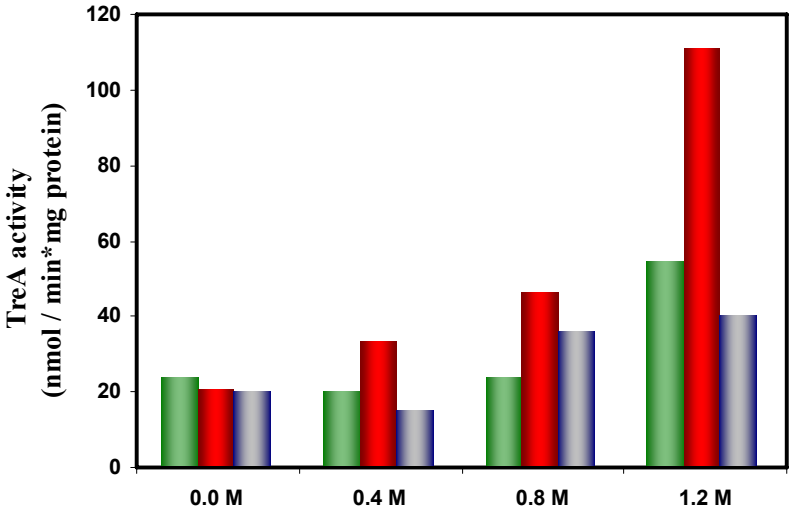

Fig. 37. TreA activities of the *yomL* '- *treA* fusion

TreA assay was performed with strains THB414 (■), THB314 (■) and THB214 (■) bearing a *treA*-fusion with the *yomL* promoter region. Overnight cultures were grown in Helmann medium without salt and subsequently inoculated in Helmann medium with the indicated NaCl concentrations. The data present the measurements obtained from two independently grown set of cultures.

6.2.5. Activities of the *yqxIJ* operon region

In the case of *yqxIJ* operon, TreA assay contributed the ambiguous results from the Northern analysis to be clarified. For this purpose a fragment containing the promoter region of *yqxI*, its coding region and the promoter region of *yqxJ* was used (Fig. 38). A very strong induction in the presence of higher amounts of DegU~P was observed. In parallel to that, in the wild type strain there was a basic level of enhancement which also followed the increase in the environmental osmolarity. And finally, when both genes encoding the DegS-DegU two-component system were deleted from the chromosome, no activity could be detected.

Taken together, the performed TreA assay of the nine genes described above fully confirmed and clarified their expression profile observed in the Northern hybridization studies. It was demonstrated that they are under the control of the DegS-DegU system and also salt induced. The rest of the investigated DegU~P targets did not fulfil these both conditions or the obtained results for some of the genes were ambiguous because of the difficulties to get clear signals after the Northern hybridization. For this reason it might be useful in parallel to the RNA technique, another method to be used for reliable data.

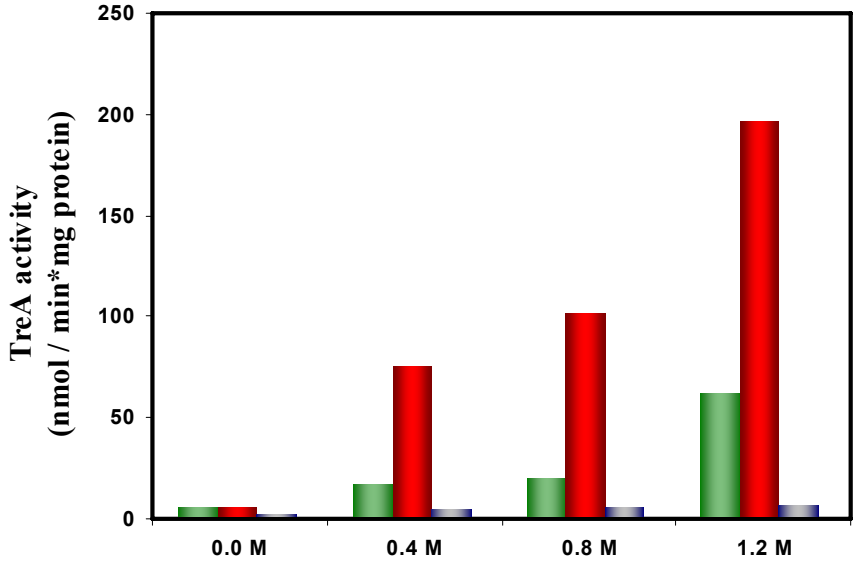

Fig. 38. TreA activities of the *yqxIJ* '- *treA* fusion

TreA assay was performed with strains THB415 (■), THB315 (■) and THB215 (■) bearing a *treA*-fusion with the *yqxIJ* region. Overnight cultures were grown in Helmann medium without salt and subsequently inoculated in Helmann medium with the indicated NaCl concentrations. The data present the measurements obtained from two independently grown set of cultures.

V. Discussion

As a soil bacterium *B. subtilis* is faced and has to cope with frequently changing parameters in its environment. Fluctuations in water availability as a result of periodically drought or flooding of the habitat threaten the cell with dehydrations under hypertonic or bursting under hypotonic conditions. To overcome these deleterious effects *B. subtilis* has evolved various systems which allow the organism to screen the environment and transduce this information to the cell apparatus. Consequently the cell can maintain its functions in accordance with the variable conditions in the surrounding habitat. In the case of sudden osmotic upshock *B. subtilis* triggers the transient induction of the entire σ^B regulon (Völker et al., 1999; Petersohn et al., 2001). In parallel, under conditions of high osmolarity the bacterium counteracts the efflux of water out of the cell by accumulation of compatible solutes via transport from the environment or by *de novo* synthesis (Bremer, 2002; Holtmann et al., 2004; Bremer and Krämer, 2000). *B. subtilis* possess five transport systems for acquiring compatible solutes (OpuA to OpuE) but the mechanism that leads to the transcription activation of the respective structural genes remain elusive so far (Horn et al., 2005; Nau-Wagner et al., 1999; Kappes et al., 1996; Spiegelhalter and Bremer, 1998). The most frequent bacterial sensory systems which detect the changes in the environement and are responsible for the appropriate responses are the two-component regulatory systems (West and Stock, 2001; Wolanin et al., 2002). From the 36 histidine kinase and 35 response regulators detected in *B. subtilis* (Kobayashi et al, 2001), only the DegS-DegU two component system was implicated in salt-induced response of the bacterium (Steil et al., 2003). The present work describes the involvement of this system in the osmotic regulation of *B. subtilis* at both transcriptional and protein level.

1. DegS-DegU two-component system is transcriptionally activated in a high salt environment

The data presented here show that the expression of structural genes of the DegS-DegU two-component system is related to the osmotic stress in the environment. To assess the role of *degS* and *degU* genes in hyperosmotic surroundings, their expression pattern was explored in strains bearing the wild type genes, the hyper *degU* allele, and in a knockout strain where both genes were deleted from the chromosome. The Northern analysis revealed accumulation of higher amounts *degS*-specific mRNA transcript when the cells were grown in high osmotic

conditions, which is consistent with the previous observation from the microarray experiment (Steil et al., 2003). This hybridization analysis confirmed also that both *degS* and *degU* are transcribed together as an operon (Fig. 9B), a situation that is usually observed for the histidine kinase and cognate response regulator pairs. In addition to the operon structure and higher transcription upon high salt, the Northern analysis performed with a separate *degU*-specific RNA probe demonstrated the presence of a second promoter within the *degS-degU* operon (Fig. 9C). Indications for the existence of such an internal promoter were reported in the past whereby after deletion of the region upstream of *degS* there was still some minor activity detected in the *lacZ* fusion measurements (Msadek et al., 1990). The Northern analysis in the current study demonstrated this clearly. Obviously, this second promoter was responsible for the sole transcription activation of the *degU* gene which was calculated from the size of the detected transcript. Usually the ratio between histidine kinases and cognate response regulator proteins in the cell is in a favour of the regulator. Hence, to accomplish the demand of higher response regulator concentrations the cell has evolved such a mechanism ensuring the transcription of the structural gene. The Northern blot technique together with the comparison of the transcription profile between the wild type, hyper and deletion mutant strains gave an opportunity not only to visualize the possible operon structure and induction ratio but also a possible influence of both gene products on their own promoters. Thus, a positive autoregulation loop from the phosphorylated DegU was observed since a higher level of the *degU*-specific mRNA transcript could be detected in the hyper mutant strain. It is the phosphorylated protein rather than the unphosphorelated one that induces the transcription from its own promoter because in the applied mutant strain the DegU~P has a seven fold higher stability (Dahl et al., 1992) than the wild type protein and consequently this form predominates in the strain. Interestingly, the level of the mRNA corresponding to the *degS-degU* operon also was slightly higher in the hyper strain than in the wild type and the same conclusion should be drawn also for the main promoter of the system. However, the higher amount of the *degS-degU* transcript was ambiguous and further promoter fusion assays declined the hypothesis for the positive autoregulation mechanisms of that main promoter.

To exclude the possibility that the observed higher mRNA levels in the hyper strain as well as upon osmotic shock were due simply to a higher stability of the isolated mRNA, further experiments were performed. They included the investigation of the transcriptional activity of *degS* and *degU* gene by means of promoter fusion assay. These assays allowed direct measurements of the promoter activities and are more reliable with comparison to the work with isolated RNA which appears to be unstable for some genes at least. The performed *treA*-

fusion assays confirmed that the promoter upstream from the *degS-degU* operon is indeed stimulated when the cells are faced with hypertonic environmental conditions. Moreover, the corresponding promoter showed higher induction levels when the cells were subjected to a sudden increase in the surrounding salinity as well as elevated activity which correlated to the magnitude of the applied NaCl concentrations (Fig. 11, 12). These results demonstrate that *B. subtilis* is able to sense not only the hypertonicity in its surrounding but also can detect the difference in the increased levels of the salinity and by this way to adjust its cellular functions with respect to the altered environmental conditions. The behaviour of the *degSU* promoter indicates that the genes which are under the control of the system and corresponding processes in which they are involved are important for both circumstances of a sudden and prolonged exposure to high salt. Glycine betaine is the most powerful osmoprotectant in many bacterial and plant cells (Felitsky et al, 2004; Waditee et al., 2005). Its accumulation in the cell could bypass the need of other osmoprotectants and is sufficient to ensure the survival of the organisms under high osmolarities. The expression of osmoregulated genes in *E. coli* was shown to be diminished in the presence of glycine betaine (Lucht and Bremer, 1994). It was shown to have the same influence on the endogenous synthesis of the osmoprotectant ectoine in *B. pasteurii* and completely prevented it (Kuhlmann and Bremer, 2002). In *B. subtilis* similar observations were reported. The transcription of structural gene of the ectoine transporter OpuE was strongly reduced after an osmotic upshift with 0.4M NaCl when glycine betaine was added in the growth medium (Spiegelhalter and Bremer, 1998). Proline is the major organic osmolyte synthesized in *B. subtilis* under elevated osmotic conditions. Nevertheless, its accumulation is repressed when the glycine betaine is transported into the cell (Whatmore et al., 1990). Such repression through glycine betaine could be demonstrated also at the transcriptional level. The transcription from the structural genes which are responsible for the *de novo* accumulation of proline under osmotic stress, *proH* and *proJ*, was greatly decreased when the cultures were supplied with 1mM glycine betaine (Brill, 2001). Interestingly, in the case of *degS-degU* promoter the addition of this strong osmoprotectant seems not to have such an influence. The transcription of both genes is retained at same level despite of the presence or absence of 1 mM glycine betaine in the medium (Fig. 12). This finding testify that the expression of *degS* and *degU* is not induced as a result of sensing high osmolarity per se but rather it responses to some specific stimulus which is a consequence of the increased environmental osmolarity and is not influenced by the presence of glycine betaine.

The expression of the *degS-degU* operon in *B. subtilis* is consequence of an osmotic effect rather than a salt-specific response, since both ionic and non-ionic osmolytes trigger the induction of the genes (Fig. 13). The induction rate in the presence of not charged compounds was found to be slightly higher for unknown reasons.

Fully consistent with the gene fusion assays are the results from the primer extension analysis where the promoter upstream from the *degS-degU* operon showed a higher induction when the cell were supplemented with 1.2M NaCl (Fig. 10). The experiment confirmed also previous data demonstrating that the corresponding region upstream from the operon is recognized by the main vegetative sigma factor from *B. subtilis* – σ^A. The same factor was shown to be responsible for the transcription of other osmotically regulated systems in *B. subtilis* like structural genes of the compatible solute transporters *opuA*, *opuB*, *opuD* and *opuE* (Kempf and Bremer, 1995; Nau-Wagner et al., 1999; Kappes et al., 1996; Spiegelhalter and Bremer, 1998). Unfortunately, the transcription start site and a putative transcription factor recognizing the internal promoter region upstream from *degU* gene could not be identified in the current study for some technical reasons.

The second assessment that was figured out from the Northern data concerning the putative autoregulation loop from the response regulator was confirmed with the *treA*-fusion assays as well. The much stronger induction of the promoter in the presence of higher DegU~P concentrations in comparison with the wild type level of the protein demonstrated with no doubt the contribution of the latter to its own promoter activation. It seems that it is the sole regulator of that internal promoter since in the absence of DegU~P no activity could be detected (Fig. 15). Obviously the same is not true for the main promoter of the system located upstream from *degS* gene. The activation from that promoter in response to the elevated external osmolarity is not correlated to the presence or absence of the gene products (Fig. 14). This contradiction with the Northern data could be attributed to the instability of the respective RNA for example and for this reason the enzyme assay used for calculating the promoter activity is considered in the study.

Altogether, the Northern blot experiments performed with the structural genes of the DegS-DegU two-component system together with promoter-fusion assays revealed that (i) both genes are transcribed together as an operon, (ii) an internal promoter is present within the operon upstream from the *degU*, (iii) elevated levels of mRNA transcripts from both genes were present in cells subjected to hypertonicity by the addition of 1.2M NaCl, (iv) there is a positive feedback mechanism from the phosphorylated DegU protein to the internal *degU*

promoter but not to the main promoter of the system, (v) induction of the *degS-degU* promoter is an osmotic rather than a salt-specific effect.

2. Significance of DegS-DegU two-component system

When the cells are challenged with increased osmolarity they adjust their cellular functions in accordance with the new situation. All above-mentioned experiments demonstrated that somehow the DegS-DegU two-component system contributes for the better adaptation of the bacterium since its transcription is stimulated under those conditions. To clarify how important is this system for the *B. subtilis* physiology and to what extent it contributes to the cell viability some growth experiments and biochemical assays were applied. It was already reported that the DegS-DegU system is involved in the protease production. The bigger halo around the hyper mutant strain detected in the protease assay clearly showed the contribution of the DegU~P to that process (Fig. 6A). Surprisingly, at the same time when the genes of the system are deleted from the chromosome, the production of exoenzymes was not completely abolished (Fig. 6C). The halo was still smaller when compared to the wild type but there was not a drastic difference. This implies that the positive regulation of the protease production must be adopted by some other factors. Indeed there are several other reviews which implicated some other genes to be involved in the positive regulation of secreted enzymes. For example overproduction of the *degR* and *degQ* genes appears to be directly responsible for the phenotype of the exoenzyme overproduction (Amory et al., 1987; Tanaka et al., 1987). Also the *senS* gene was defined to be responsible for the production of extracellular proteases (Wang and Doi, 1990). Other pair of genes involved in the degradative enzymes regulation are *tenA* and *tenI*, respectively (Pang et al., 1991). Nevertheless, for all of these genes it was shown that they lost their positive protease induction when expressed in strain with deleted *degS* and *degU* genes. On the other hand they were found to be dispensable for the viability of *B. subtilis* since the deletion of each of them did not show some significant phenotype. Nevertheless, one could imagine that all of these genes are somehow interwoven in a complex network responsible for the degradative enzymes production and when one regulator is missing its function is occupied by the others. On the other hand it might be possible that some of the genes encoding degradative enzymes are under the control of additional factors other that DegU. For example it was shown that the expression of *sacB*, which encodes levansucrase, is under the control of two independent regulatory mechanisms – the *degS-degU* system and induction by sucrose (Klier et al., 1987; Crutz and Steinmetz, 1992). Another

example is the expression of *degQ* gene. In addition to DegS-DegU system, it appears to be affected by three other regulatory systems: ComP-ComA two component system, which is involved in controlling competence gene expression, catabolite repression and regulation by phosphate (Msadek et al., 1991). Since the ComP-ComA two-component system controls the *degQ* expression and the *degQ* in turn, controls the expression of degradative enzymes, one would expect that at least under some conditions, expression of these enzymes would also be affected by the ComP-ComA pair.

To clarify the importance of the DegS-DegU two-component system for the cell physiology some growth experiments in different genetic backgrounds were carried out. The results demonstrated that the system gives some advantage to the cell subjected to high osmotic conditions since their growth is clearly improved in the presence of *degU* hyper allele. On the other hand, the chromosomal inactivation of *degS* and *degU* genes did not result in any significant phenotype with respect to high osmolarity of the medium. One of the possibilities that could explain this phenomenon is the assumption that the expression of the genes controlled by this regulatory pair is also induced by some other regulators which can compensate the lost of the *degS* and *degU*. And of course the cell has other meanings to overcome the hyperosmotic shock. As it is known, when *B. subtilis* is faced with unfavourable conditions including high osmolarity, it has the ability to accumulate large amounts of compatible solutes via *de novo* synthesis or transport from the environment (Bremer, 2002; Bremer and Krämer, 2000; Holtmann et al., 2004). In parallel, under such growth restricting conditions *B. subtilis* activates transiently the σ^B regulon which encompasses many genes and also contributes for the survival of the bacterium at high salt. As it can be seen the cell has evolved many molecular mechanisms to ensure its normal function in the constantly changing environment and the idea that disruption of one of those mechanisms would disturb its viability is not very probable. One such an example is the regulation of genes involved in competence development. In addition to *degU* and *comA* products, several other genes are known to be involved in that process, like *srfA*, *mecA*, *mecB*, *comK* (Msadek et al., 1991). It was demonstrated that the expression of late competence genes could be restored by *mecA* or *mecB* mutations in strains carrying *degU* or *comA* disruptions (Roggiani et al., 1990). Altogether the growth experiments and transcriptional investigations of the *degS-degU* operon from *B. subtilis* revealed that the system is essential for the adaptation to elevated osmolarities but its deletion is not sufficient to confer a considerable phenotype under the applied experimental conditions.

As a matter of fact, the majority of the two-component systems detected in *B. subtilis* were found to be not essential for growth since the deletion mutants of their response regulators did not show any influence on the colony morphology, growth or sporulation (Kobayashi et al., 2001). This indicates that perhaps most of the two-component regulation is used to enhance the versatility of the responses of an organism to environmental stimuli through regulation of normally unexpressed genes.

3. Expression of the DegU regulon upon osmotic shock

For better understanding the role of DegS-DegU pair, identification of the genes which are under the control of the system would be of a great help. Up to know there are two reviews that concern the putative target genes of the DegU protein. Ogura and co-workers took the strategy of identifying DegU regulon by using a DNA microarray analysis. For this purpose, *degU* gene was cloned downstream of a *spac* promoter (P*spac*) in plasmid pDG148 in *E. coli*, and the constructed plasmid was then introduced into *B. subtilis* strain with disruption in the cognate sensor kinase gene (Ogura et al., 2001). The cells were grown to mid-log phase and the expression of the DegU regulator gene was induced by the addition of IPTG. By this way they could identify that the expression of 116 genes (~ 2.8% of *B. subtilis* genome) was affected when DegU was overexpressed. This strategy appeared to work well for the detection of target genes not only for the DegS-DegU two-component system but also for ComP-ComA and PhoP-PhoR regulatory pairs (Ogura et al., 2001). Nevertheless, this contradicts somehow to the concept that namely the phosphorylation state of the DegU regulator discriminates between the genes to be up- or downregulated and hence controls mutually exclusive cellular processes such as degradative enzyme production and genetic competence.

The other working group studied the DegS-DegU regulon by combining proteomic and transcriptomic approaches. The authors compared the gene expression patterns of the wild type and the *degU32*(Hy) mutant (Mäder et al., 2002). The extracellular proteome and the corresponding transcriptome were analyzed in both exponential and stationary growth phase. By this way they could identified 14 extracellular proteins which were positively regulated by DegU~P. 13 of them were identified from a previous study as well (Antelmann et al., 2001). In addition, it was figured out that these proteins have different expression pattern since some of them were only weakly synthesized in the exponential growth phase and strongly induced in stationary phase. To obtain a more complete picture, they compared the expression pattern of the wild type and hyper mutant again in exponential and stationary growth phase. This

approach led to the identification of 32 transcriptional units that were under the control of DegU~P – 18 of them were stronger expressed in the exponential phase while the other 14 were upregulated in the stationary phase.

The identified DegU~P target genes from the above mentioned studies overlapped just to a certain extent. The differences in the observed transcriptional units regulated from the DegS-DegU system could be attributed to the different experimental procedures applied. Moreover, the identification of the DegU regulon in both working groups was perform without concerning the possible signal that might trigger the induction of the whole system.

The involvement of the DegS-DegU system in the osmotic response of the *B. subtilis* that was deduced from previous publications (Kunst and Rapoport, 1995; Steil et al., 2003) and this study led to a strategy where the putative downstream genes of the DegU regulator could be identified more correctly by using an osmotic stimulus. A set of 18 genes was identified by comparing the DegU regulon defined by Mäder et al., 2002, and Ogura et al., 2001, with genes that showed a higher expression level under high osmotic conditions (Steil et al., 2003). In this study the possible target genes were explored more precisely via Northern blot analysis where a specific RNA probe was applied for each separate gene and the transcription from the latter was followed in the background of the wild type, hyper and deletion mutant of the DegU response regulator. In addition, the expression pattern of the corresponding targets was followed also in the hyperosmotic background via addition of 1.2M NaCl in the growth medium. The Northern analysis demonstrated that when the cells are challenged with high salt concentrations the DegU~P enhances the mRNA level of 9 genes altogether. Among them three were newly identified to be positively regulated – these are *yitN*, *yitO* and *yitP*. The *yddT*, *yomL* and *yoaJ* are transcribed alone whereas the rest 6 genes form two operons – *yqxIJ* and *yitPONM*, respectively (Fig. 27 to 31). Besides, one operon was identified to fulfil only one of the adopted criteria. Namely, it showed higher expression level when subjected to high salt but seemed not to be under the control of DegU regulator. This operon includes the *mpr* and *ybfJ* (Fig. 32). A similar pattern was ruled out by the group of Mäder and co-workers, which found that the amount of Mpr protein, which codes for an extracellular metalloprotease, was induced to a very high extent in the background of the hyper mutant strain, and in parallel a higher expression of the respective structural gene could not be detected. They attributed these observations to the higher stability of the extracellular proteins than their short-lived mRNAs. Among the investigated genes, for yjhA, yfjB and yfjC higher accumulation of specific mRNA transcripts could be observed only in non-salt growth conditions. Hence, these genes seem to be activated by the phosphorylated DegU regulator but not upon salt

stress. The other explanation would be of course the instability of the corresponding mRNA transcripts under those experimental conditions.

Additional confirmation supporting the role of the phosphorylated DegU protein in regulating those nine genes, which fulfilled both adopted criteria, was obtained on the basis of one further experiment. The promoter activities of these genes were explored under the same conditions and it was confirmed that their expression is in accordance with the external osmolarity. Moreover, the presence of the hyper active *degU* allele triggered their induction even higher (Fig. 34 to 38). Another conclusion that can be figured out from the promoter-fusion assay is that some of the investigated genes (*yomL* and *yddT*) showed similar induction level in the wild type and the deletion mutant strain. This reflects the fact that most probably the DegU~P is not the sole factor involved in their transcription regulation.

Hence, despite of the ability to define a set of target genes which were induced from the phosphorylated DegU in the presence of high osmotic conditions, the following limitations of the present analysis should be kept in mind for any further investigation: there is a possibility that some fraction of target genes could not be expressed under the present experimental conditions due to additional negative regulation mediated by other regulatory proteins. On the other hand, for part of the investigated regulon the hybridization experiments did not lead to satisfying results, i.e. the mRNA expression level was undetectable or the expression pattern of the respective genes was ambiguous. This observation does not mean necessarily that those genes are not under DegU control. The absence of product to be seen might be due to the instability of the corresponding mRNA. Therefore, the potential problems of the Northern analysis applied in this study must be taken into consideration upon interpretation of the target genes. Hence, this part of the regulon needs definitely further investigation by means of other methods like promoter fusion assay which was applied for part of the regulon. Another possibility would be genetic analysis of the interactions between the *cis* acting elements of the target gene candidates and the DegU regulator by gel retardation assays and DNA footprinting.

The analysis of the promoter regions of DegU regulated salt-induced genes by means of the Extra Train database (http://www.era7.com/ExtraTrain/) did not lead to identification of some common motif which would be involved in their transcriptional activation. Moreover, the transcription start point of those promoters is not known which makes this analysis more difficult. Previous study revealed the *cis*-acting sequencing of some target genes which were important for their activation through DegU regulation. As it could be demonstrated, DegU recognizes tandem repeats or inverted repeats in the different promoters but the overall model

seems to be specific for each target with respect to sequence length and arrangement of the consensus motif (Fig. 41). One additional review postulated that the promoters of the DegU-regulated genes may contain DegU binding sites with different affinities (Kobayashi, 2007). Therefore, the localization of the DegU target motifs must be performed with more precise methods like DNA footprinting.

The detected transcriptional activity of some of the investigated gene targets in the absence of the DegU regulator implies that these genes are not solely induced by the DegS-DegU two-component system. This suggests that there should be independent regulator acting on the same targets. It is more reasonable for one to thing that the overall tolerance to high salinity stress is a coordinated action of multiple stress responsive genes, which also cross talk with other components of stress signal transduction pathways.

Indeed it was demonstrated that both DegS-DegU and ComP-ComA two-component regulatory systems were involved in degradative enzyme synthesis and competence development in *B. subtilis* (Kunst et al., 1994). Moreover previous studies have already noticed such a scenario for some other two-component systems form *B. subtilis*. A striking overlap was observed for the target genes of the YxjM-YxjL and YvqE-YvqC systems, where out of 19 gene candidates upregulated by YxjM-YxjL, 17 were included also in the proposal regulon of YvqE-YvqC. Such an overlap was also observed for the DesK-DesR and YvfT-YvfU systems (Kobayashi, 2001).

Many of the DegU controlled genes are also regulated by other factors. Typical examples are *aprE* and *degQ* genes. The expression of the alkaline protease (AprE) is regulated by a complex network of activators and repressors that includes the products *degU*, *spo0A*, *degQ*, *sinR*, *salA*, *hpr* (*scoC*), *abrB* (Henner et al., 1988; Olmos et al., 1997; Ogura et al., 2004). In the promoter region of *degQ* were detected separate targets required for regulation from DegS-DegU and ComP-ComA system, respectively (Msadek et al., 1991). The *wapA* gene which is downregulated under high salt from the phosphorylated DegU is obviously regulated by other factor since a residual negative control was observed in the absence of DegU protein under the same experimental conditions (Dartois et al., 1998).

Many of the target genes of Spo0A and ResD for example, are also known to be regulated by other regulatory proteins (Fawcett et al., 2000; Ye et al., 2000).

However, at that state of this work, it was not shown, whether the DegU regulator directly mediates the expression regulation of the postulated target genes. Another point that could not be clarified is how exactly the products of the detected target genes contributed to the better adaptation of *B. subtilis* under high osmotic conditions. Since the nature of the corresponding

proteins is unknown, their function could not be attributed to certain cellular processes which might be implicated in the osmostress response.

4. Sensing properties of the DegS kinase

In bacteria, at least two types of osmosensors were found. On one hand, transport systems can exhibit osmosensory properties, which are directly regulated at the level of activity. On the other hand, osmosensors can mediate the transcriptional regulation of osmoregulated transport systems or other cellular functions in order to ensure the viability of an organism, and thus act at the level of expression. However, these transcriptional regulators themselves are regulated at the level of activity. The latter case can be accomplished by two-component systems, as seems to be the case for DegS-DegU system. After elucidation the transcriptional activity of the *degS-degU* genes, the next step was to clarify the behaviour of the corresponding proteins upon high osmolarity. The osmotic activation of DegS may be triggered directly by a variety of parameters including a change in cell turgor, a change in the internal osmolarity, changes in the hydration state of DegS as a consequence of altered internal osmolarity, changes in the cytoplasmic ion concentration, or changes in the concentration of specific compounds interacting directly with the sensor kinase. The cytoplasmic location of the DegS protein allowed the investigation of its sensing properties via development of an *in vitro* phosphorylation assay where the activation of both proteins could be easily followed by the use of radioactively labelled ATP.

The described *in vitro* system does not allow the variation of turgor pressure. However, in case of the well described osmosensors BetP of *C. glutamicum*, ProP and KdpD of *E. coli*, or OpuA of *Lactococcus lactis*, the cell turgor as signal triggering protein activity was shown to be an unlikely stimulus (Morbach and Krämer, 2002; Culham *et al*., 2003; Jung and Altendorf, 2002; Poolman *et al*., 2002).

In order to analyze which stimulus is used by DegS to detect hyperosmotic stress, the *in vitro* effect of various possible osmostress-related signals was tested. First, a variation of monovalent ions was performed, to discriminate between the influence of ionic strength or specific ion effects. The presence of higher concentrations of KCl, NaCl and RbCl did not lead to stimulation of the DegS autophosphorylation (Fig. 21). On the contrary, the addition of higher amount of these substrates seems even to decrease the phosphor-accepting activities of the kinase. Since in all cases the same anion was applied, the observed suppression of the activity is attributed to Cl^-.

In case of membrane-bound osmosensors, a number of proteins have been shown to be able to sense specifically intracellular monovalent ions or the internal ionic strength in general. It could be demonstrated that changes in the internal cellular conditions are measured from compatible solute uptake carriers. For example, the Na^+/betaine carrier BetP of *C. glutamicum* was reported to specifically sense internal K^+ (Rübenhagen *et al*., 2001; Schiller *et al*., 2004), whereas the compatible solute transporter ProP of *E. coli* was postulated to be stimulated not only by internal cations, but additionally by cytoplasmic macromolecules (Racher *et al*., 2001; Culham *et al*., 2003). In case of the reconstituted ABC transporter OpuA of *L. lactis* an activation by increased internal ionic strength, which is thought to result in perturbations in the ionic interactions between protein and the surrounding membrane system, was proposed (van der Heide *et al*., 2001). In contrast, the sensing of internal conditions by two-component systems was clearly shown only for the histidine kinases KdpD of *E. coli* and MtrB of *C. glutamicum*. The KdpDE system regulates the transcription of the *kdpFABC* operon, encoding the high affinity K^+ uptake ATPase KdpFABC (Polarek *et al*., 1992; Walderhaug *et al*., 1992). This two-component system is related to two distinct *in vivo* conditions: the *kdpFABC* expression is regulated in response to low K^+ concentrations as well as to an osmotic upshift. Analysis of KdpD in right-side-out membrane vesicles showed that the histidine kinase senses two sets of stimuli *in vitro*. Whereas internal K^+ at concentration of 1 mM, imposed an inhibitory effect on KdpD activity, an increased internal ionic strength achieved by NaCl, RbCl, or HEPES-Na, had an activating effect on this protein (Jung *et al*., 2000). The MtrB/MtrA two-component system from *C. glutamicum* was recently shown to be involved in the osmostress response of the bacterium. It was demonstrated that both membrane shrinkage and the presence of high concentration of different chemical compounds led to the activation of the MtrB sensor kinase (Möker et al., 2007). The observed broad substrate specificity led to the suggestion that these compounds are involved in changing the hydration state of the kinase rather than binding to a specific binding site and by this way triggering its activation. Also in *E. coli*, the EnvZ-OmpR two-component system was reported to regulate the transcription of *ompC* and *ompF*, encoding porins of the outer membrane, in dependence of the medium osmolality (Tsung et al., 1989). Both the sensor kinase EnvZ and response regulator OmpR of this system were reconstituted and a stimulatory effect was reported for the monovalent ions K^+, Rb^+, NH_4^+, and Na^+, with highest influence in presence of K^+ (Jung et al., 2001).

To investigate further the sensing properties of the DegS kinase, the influence of various solutes on its autokinase activity was monitored. When the osmolarity was raised by the

addition of different ionic and non-ionic compounds, a substantial increase in the DegS autophosphorylation was detected in the presence of Na glutamate (Fig. 22). Glycine betaine and proline, which are known to be accumulated upon exposure to elevated osmolarities, did not lead to any significant enhancement of DegS~P. Glucose, sucrose and lactose also failed to stimulate the kinase activity. The slight increase of the radioactive signal in the presence of sucrose is rather unspecific effect because *B. subtilis* does not accumulate this sugar and consequently it could not serve as a direct signal for the DegS kinase. This *in vitro* experiment points towards the fact that the activation of the DegS trough phosphorylation is not a result of the increased osmolarity per se but is rather a specific response to a certain compound, in this case Na glutamate. To test further this hypothesis the kinase activities were explored in the presence of K- and Na glutamate, respectively, and the specific influence of the glutamate was confirmed since there was no difference with respect to the available cation (Fig.23). Moreover, when different K- and Na salts were tested for their possible influence on the DegS kinase activities, no significant autophosphorylation could be detected (Fig. 24). DegS protein was found also to be phosphorylated in dependence of the applied K glutamate concentrations (Fig. 25). This implies that the kinase is able to sense specifically the glutamate content and to adjust its rate of phosphorylation in accordance to the amount of the available amino acid. Since all received information is based on the *in vitro* studies of the DegS-DegU system, the question that arises is if these effects could be attributed to the situation *in vivo*. It is known that generally the glutamate content in *B. subtilis* is high (Whatmore et al., 1990). The amino acid pool measurements of cells subjected to a sudden osmotic shock revealed that the main compound that is accumulated under these conditions is proline. The glutamate accumulation in parallel is increased only about a factor of two (Whatmore et al., 1990). Nevertheless, it has to be mentioned that the earliest time point at which the authors have measured the changes in the amino acid pool was two hours after the osmotic upshock. In the currents study, the internal glutamate content of *B. subtilis* cultures was measured upon sudden osmotic challenge but the analysis was performed with respect to the immediate response of the cells, i.e. the amino acid pool was explored within the first minutes up to two hours after the shock. This revealed a sudden increase of the glutamate concentrations in the first 30 min after the NaCl treatment and a following decrease afterwards. Altogether, the level of the glutamate was kept relatively constant after these 30 min and the values were still higher than those observed before the shock (Fig. 26). These data do not contradict to the observed slight increase in the glutamate content from Whatmore and co-workers (Whatmore et al., 1990), but rather complement it. Since the cultures that were used for the measurements were grown

in medium supplemented with glutamate, the following question is where the glutamate comes from – is it transported from the environment or is it synthesized within the cell. Hence, an experiment performed in medium not supplemented with glutamate would shed some light on that point.

5. Is the glutamate the requested intracellular signal sensed by DegS kinase?

The accumulation of glutamate as an osmoprotectant has been demonstrated in many bacterial species. For example the sequence of events following an osmotic upshock is well demonstrated in *E. coli*. When the cells are grown at elevated osmolarities they accumulate large amount of K^+ as a primary response. Concomitantly, to prevent the alkalization of the cytoplasm, the glutamate is produced as a counter-ion (Dinnbier et al., 1988; Cayley et al., 1991). It has been postulated that the potassium glutamate typically stabilizes DNA-protein interactions compared to equivalent chloride salt (Leirmo et al., 1987). Nevertheless, higher concentrations have the capacity to disturb cellular metabolism (Gralla and Vargas, 2006), making potassium glutamate a less preferred osmolyte for long-term protection against hyperosmotic shock. For *E. coli* it was shown that approximately 30 min after the salt shock the cells start to synthesize trehalose, and after additional 2 hours they replace it by other osmoprotectants like proline (Dinnbier et al., 1988). This initial accumulation of glutamate and the following reduction after half an hour reminiscent very much the situation observed in the current study. However, Dinnbier and co-workers reported the same behaviour for K^+, i.e. after 30 min the cells started to release the accumulated ions. In *B. subtilis* the K^+ pool was shown to increase from the basal level of 350 mM to 650mM, respectively, but the corresponding measurements were performed 2 hours after the salt shock (Whatmore et al., 1990). Hence, it would be interesting to follow the changes in the internal K^+ content immediately after the osmotic shock as well.

There are also other examples where the glutamate accumulation serves as a priming response to the elevated external osmolarities. When *Rhizobium meliloti* cultures were imposed to osmotic stress, they were shown to accumulate K^+ and glutamate. The effect was not specific to Na^+ but also occurred when the osmolarity was shifted by means of K^+, sucrose or polyethylene glycol (Botsford and Lewis, 1990). The increased production of glutamate was reported to occur 5 min after the shock, and it was indicated by use of antibiotics that *de novo* protein synthesis was not required for this process. Similar is the situation in *Salmonella typhimurium*. It was reported that when the cells are grown in minimal medium with 500 mM

NaCl, KCl or sucrose, the amount of the accumulated glutamate is increased. Moreover, strains with mutations in glutamate synthase or in glutamate dehydrogenase accumulated nearly normal levels of glutamate which demonstrated that none of these enzymes is solely responsible for the glutamate excess (Botsford et al., 1994). Identification of the glutamate as a counter-ion for K^+ was reported also by Yan and co-workers. They reported that the glutamate is required to maintain the steady-state K^+ pool in *S. typhimurium* (Yan et al., 1996). Glutamate accumulation in response to hyperosmotic stress was reported also for *Brevibacterium lactofermentum* and *Corynebacterium glutamicum* (Skjerdal et al., 1996).

In halophilic bacterium Halomonas elongata uptake of K^+ was reported to occur together with synthesis of the predominant compatible solute ectoine. Also the glutamate content changes in a similar way to the K^+ content, keeping the K^+: glutamate ratio at a value of 2.4:1. The difference with the non-halophilic bacteria is that the potassium and glutamate levels are not replaced by compatible solutes but remain elevated at least up to 120 min (Kraegeloh and Kunte, 2002).

An interesting finding was reported recently for the halophilic bacterium *Halobacillus halophilus*. At intermediate salinities corresponding to 1M NaCl, cells produce glutamate and glutamine in a chloride-dependent manner (Saum et al., 2006). Besides, the bacterium can switches its osmolyte strategy and produces proline as the dominant solute at higher salinities achieved via 2 to 3M NaCl (Saum and Müller, 2007). The proline biosynthetic genes (*proH*, *proJ* and *proA*) form an operon which was shown to be salinity dependent with maximum at 2.5M NaCl. Chloride salts led to a highest accumulation of proline but interestingly, the chloride could be substituted to a large extent by glutamate salts. The authors analysed further these findings and could demonstrate that the mRNA levels of all three *pro* genes were increased up to 90-fold in the presence of glutamate. A minimal concentration of 0.2M glutamate already could stimulate that transcription. These data demonstrated that the glutamate is involved in the switch of osmolyte strategy from glutamate to proline as the dominant compatible solute during the transition from moderate to high salinity (Saum and Müller, 2007).

These results are interesting with respect to the situation in *B. subtilis*. It is known that after an osmotic upshock the expression of the *proHJ* operon, which encodes the enzymes of the biosynthetic proline production, is stimulated (Brill, 2001) and this lead to *de novo* synthesis of this amino acid (Whatmore et al., 1990). This biosynthetic pathway leads to accumulation of proline at the expense of glutamate and as a result, the proline content is increased to ensure the cell survival under high osmolarities, and the glutamate content is decreased (Brill,

2001). This knowledge together with the observations from the current study that the glutamate concentration is increased only within the first minutes after the salt shock reminiscent the situation in *E. coli*, where the initial transient accumulation of K^+ and glutamate is later followed by trehalose production and finally replaced by compatible solutes like proline (Dinnbier, 1988). *B. subtilis* can not use trehalose as an osmoprotectant and the proline synthesis seem to be the second step of its osmoadaptation where it replaces the glutamate. The findings that glutamate functions as an inducer of the *pro* operon from *H. halophilus* upon high salt challenge, could be true also for the *proHJ* operon in *B. subtilis*. Moreover up to now there is not a clue what leads to the expression of these genes. The investigations for some possible transcriptional regulators that might influence the osmotic induction of the *proHJ* promoter could not be identified (Dolezal, 2006).

A promising hypothesis could include the following series of events: upon elevated osmolarities *B. subtilis* accumulates K^+ and glutamate as initial transient response. This increased glutamate content would lead to autophosphorylation of the DegS kinase which in turn donate the phosphor to its cognate response regulator DegU. The activated regulator then can fulfil its function by induction of the downstream genes, respectively *proHJ*. The expression would finally lead to the activation of ProH and ProJ, which contribute for the *de novo* production of the more potent osmoprotectant proline at the expense of glutamate. Unfortunately, some of the experimental data do not support such a hypothesis – up to now the *proH* and *proJ* genes were not reported as belonging to the DegU-regulon.

Another weak point is activation of the DegS kinase through glutamate. Here, the activation through phosphorylation could be demonstrated very clearly for the DegS protein. On the other hand, the induction of the whole phosphorylation cascade including DegU was attained only in the presence of glutamate. This still support the possible role of the glutamate but does not explain the fail of the phosphotransfer in its absence, which was reported in the literature (Mukai et al., 1990; Dahl et al., 1991, 1992; Tanaka et al., 1991). Most probably it is due to a lower activity of the response regulator. All the experiments from the current study point to the fact that the glutamate could serve as an internal signal which can triggers the transduction cascade from DegS to DegU and finally transcription regulation of the respective downstream genes. There is another fact supporting the possible role of the glutamate. When the osmotic upshift is performed with cultures grown in SMM medium, the corresponding mRNA levels of *degU* gene revealed almost no difference compared with that under non-osmotic conditions. On the contrary, when the same measurements were performed in Helmann medium, which is used also in the current study, the accumulation of mRNA transcripts was

higher in cells subjected to high osmolarity in comparison to the low salt growth conditions (T. Hoffmann, personal communications). The observed difference in the induction levels could be attributed to the presence of glutamate in the Helmann medium and not in SMM. However, this speculation need to be further explored since both media differ also by other components. Despite of being very good hint for the role of the glutamate, the *in vitro* studies with the DegS-DegU system must be further investigated. The reconstruction of the whole in vitro cascade would be necessary, so that the specific influence of the glutamate to be demonstrated for both proteins. Additionally, *in vivo* studies also would contribute for clarifying the role of the glutamate.

Even if one accepts that the glutamate is the intracellular signal input for the DegS kinase, there still remains one question, namely, how exactly the activities of the HK core are regulated by the presence of the glutamate.

6. Molecular mechanisms involved in the DegS-DegU regulation

Despite of the characterization of the osmotically induced DegS-DegU two-component system at the transcriptional level and the positive regulation of some target genes there is still a question remaining – what are the molecular mechanisms which regulate the system.

The first level of regulation concerns the transcription of *degS* and *degU* genes. Then come the mechanisms which are involved in the regulation of the DegS and DegU proteins, and finally, how exactly these proteins exert their effect on the respective downstream genes.

It was clarified that the structural gene of the DegU response regulator is induced in a feedback mechanism and obviously no other factor is involved in that process. On the other hand, nothing is known about the transcription regulation of the histidine kinase structural gene. Most probably some other transcriptional factor must be involved in this process but it remains elusive so far. Some gel retardation assays with total cell extract could shed a light on the possible role of such a regulator. Another possibility for the transcriptional activation of the *degS* could involve some changes in the DNA supercoiling. Indeed, it has been reported that an osmotic upshift of 0.5M NaCl triggers an increase in the negative supercoiling of plasmid DNA within a few minutes (Hsieh et al., 1991). One further example was demonstrated for the transcription of the *osmE* gene from *E.coli*. In a medium of low osmotic pressure, expression of *osmE* is induced at the onset of stationary phase. At elevated osmotic pressure, a biphasic induction pattern was observed. The first step occurs during exponential phase, and this is followed by a strong induction at the onset of stationary phase. Both steps

appear to result from stimulation of transcription at the same promoter, *osmE*$_p$. In the absence of σ^S, the stationary phase sigma factor encoded by *rpoS*, *osmE*$_p$ stationary phase induction is abolished, while the osmotic effect is still observed. The authors postulated that changes in DNA supercoiling are involved in the osmotic induction of *osmE*$_p$ (Conter et al., 1997).

Regulation at the protein level seems to be quite complicating having in mind the variety of cellular processes that are controlled by the DegS-DegU two-component system. Simple "off" and "on" phosphorylation state of the regulator being responsible for the activation of the variety of the target genes seems a very plain model. More acceptable is the situation where the DegU control is more flexible and has the capacity to integrate physiological responses along a gradient of DegU phosphorylation. Indeed, there is a report showing that: (i) the swarming motility of *B. subtilis* is activated by very low levels of DegU~P that can be generated independently from its cognate sensor kinase DegS; (ii) the complex colony architecture is activated by low levels of DegU~P that are produced in a DegS-dependent manner to activate transcription of *yvcA*, a novel gene required for complex colony architecture; and (iii) high levels of DegU~P inhibit complex colony architecture and swarming motility but are required prior to the activation of exoprotease production (Verhamme et al., 2007). Additional report also supported the findings that the level of DegU phosphorylation may determine the temporal regulation of DegU-controlled genes (Kobayashi, 2007). The author postulated that low levels of DegU~P are necessary for swarming whereas high levels are required for biofilm formation and downregulate the motility of *B. subtilis*.

Hence, such differences at the level of phosphorylation imply that additional factors must be involved in order to coordinate this fine tuning of the DegU activity. It was demonstrated that one of the positive regulators of the exoenzyme production, DegR, exerts its effect by directly influencing the DegU protein. In vitro studies revealed that in the presence of DegR higher phosphorylation of the DegU could be observed. The authors defined that DegR does not stimulate the DegS autophosphorylation but rather retarded the rate of DegU dephosphorylation (Mukai et al., 1992). One hypothesis for this stabilizing effect of DegR suggests that the N-terminal region of DegS is involved in the dephosphorylation of DegU phosphate and that in some way DegR inhibits this reaction by competition (Ogura et al., 1994). The same authors also reported that the *proB* gene, encoding γ-glutamyl kinase, plays a role in enhancing alkaline protease synthesis in a DegS-dependent manner. They suggested that the accumulation of γ-glutamyl phosphate in the cell may lead to higher levels of

phosphorylated DegU, either by directly acting as a high-energy acylphosphate phosphodonor for DegS or by regulating the phosphatase activity of DegS.

Another protein reported to be involved in the regulation of DegS-DegU phosphorylation cascade is DegQ. This protein is involved in the regulation of degradative enzyme production (Amory et al., 1987) and its expression is controlled by DegS-DegU system (Msadek et al., 1991). In parallel, DegQ protein is involved in regulation of the phosphorylation cascade similar to that of DegR. Purified DegS~P was incubated with DegU in the presence or absence of DegQ, and the phosphorylated proteins were analysed over time. It was shown that the intensity of the DegU~P signal is much stronger in the presence of DegQ than in its absence. These observations indicate that DegQ enhances phosphotransfer from DegS~P to DegU (Kobayashi, 2007).

On another level, it can not be excluded that the phosphorylation state of DegS and DegU proteins could be influenced by some other two-component system. By this way distinct signalling pathways can be integrated into cellular networks. One example of this integration is between the pathways controlling phosphate utilization (PhoR/PhoP), aerobic and anaerobic respiration (ResE/ResD), and sporulation (KinA-B/Spo0A). Respiration and phosphate utilization are co-regulated; phosphorylated PhoP is required for expression of ResD and vice versa (Sun et al., 1996; Birkey et al., 1998). Furthermore, once the cell commits the sporulation, respiration and phosphate utilization are down-regulated. Phosphorylated Spo0A is a negative regulator of both ResD~P and PhoP~P, and therefore mutually exclusive with both of these responses (Sun et al., 1996; Hulett, 1996).

Also one can not exclude phosphorylation of DegU protein by another histidine kinase due to a phenomenon described as “cross-talk” (Drepper et al., 2006; Mukhopadhyay and Varughese, 2005). Structural and functional conservation of two-component proteins suggests the potential for phosphotransfer between noncognate pairs. Although such transfer is commonly observed in vitro (Fisher et al., 1995), it appears to be rare in vivo. Nevertheless, there are few cases where such a cross regulation could be demonstrated. The EnvZ-OmpR two-component system is known to be involved in the regulation of the outer membrane porins OmpC and OmpF at high or low osmotic conditions, respectively. Matsubara and co-workers showed that under anaerobic growth conditions the *arcB* gene, encoding the anaero-sensory histidine kinase, appears to be an auxiliary genetic determinant that regulates the expression profile of the porins. The AbrB protein was involved in regulating the porins in an OmpR-dependent manner (Matsubara et al., 2000). This model is a clear example of the interplay of two distinct His-to-Asp phosphorelay signalling pathways.

The next question that arises is how exactly the phosphorylation of the DegU response regulator stimulates the transcription of its downstream genes. As it was already discussed the phosphorylated form of DegU is necessary for the overproduction of degradative enzymes. Still there is a report indicating that the salt stress influences the expression of the levansucrase and alkaline protease genes (*sacB* and *aprE*, respectively) in a totally different way in DegU dependent manner. Namely, the *sacB* promoter is induced when 1M NaCl or 1M KCl are present in the medium while the transcription from the *aprE* promoter seem to be strongly decreased under those conditions (Kunst and Rapoport, 1995). This situation is reminiscent to that of *ompC* and *ompF* genes from *E.coli* which are under the control of EnvZ-OmpR system. The *ompC* is preferentially expressed under high osmolarity whereas *ompF* is repressed under those conditions but is induced at low osmolarities. For the expression of both targets, phosphorylated OmpR is required. Mutational analysis of the carboxy-terminal phosphorylation domain implies that the OmpR-phosphate must adopt different confirmations when binds at *ompF* and *ompC* promoter regions (Mattison et al., 2002).

However, there are other probabilities which could lead to differential expression of the corresponding target genes. The expression of degradative enzymes in *B. subtilis* is under the control of many regulatory proteins which may probably interfere with this phenomenon. It is possible that the salt stress signal is transduced by the DegS-DegU system, leading to positive or negative effects, depending on the involvement of additional regulatory genes. For example, it is possible that salt stress leads to dephosphorylation of the response regulator Spo0A and by this way preventing both sporulation and *aprE* expression (Ferrari et al., 1988). Alternatively, the binding of DegU protein to its target promoters could be regulated in different manner. One possibility is the effect of the Rap-Phr systems. The Rap phosphatases are a conserved family of regulatory proteins. Pairs of *rap* and *phr* genes usually constitute operons, each of which encodes a pair of interacting regulatory factors that modulate the phosphorylation state of specific response regulators within two-component signal transduction systems (Pottathil and Lazazzera, 2003). In *B. subtilis* genome there are seven operons coding for Rap-Phr and four Raps without cognate Phr (Ogura et al., 2003). It could be demonstrated that the disruption of *rapG* and *phrG* resulted in enhancement and reduction of *aprE-lacZ* expression, respectively, pointing to the fact that PhrG inhibits RapG activity. Further gel retardation assays revealed that RapG serves as an inhibitor of DegU binding to the promoter regions of *aprE* and *comK*, and this inhibition is counteracted by the Phr peptide (Ogura et al., 2003). Interestingly, it was also demonstrated that the respective structural

genes *rapG* and *phrG* were also subject of salt induction (Steil et al., 2003). This fact is an additional support regarding the role of DegS-DegU two-component system in the osmotic regulation of *B. subtilis* and shed a light on the point concerning the transduction of the information from the environment to the cytoplasmic components of the system.

Another type of regulation at the transcriptional level constitutes cooperative binding of the regulator to its target promoter. Such an effect was demonstrated for some of the DegU-controlled promoters. There were observed gradually retarded bands in gel shift assays which corresponded to the increased DegU concentrations (Kobayashi, 2007).

Hamoen and co-workers postulated additional meaning of regulation concerning DegU targets. They have investigated the role of DegU in *comK* activation. ComK is required for the transcription of genes encoding the DNA uptake, as well as for activation of its own gene. DNA footprinting revealed that DegU promoter binding site overlaps with the ComK binding site. The authors proposed that DegU functions at the initiation of competence development, when ComK concentrations are insufficient to support comK transcription. Therefore, DegU is needed to prime the autostimulatory transcription of *comK*. This activation by priming is another way by which response regulators can stimulate the transcription (Hamoen et al, 2000).

Another possibility is the regulation through the RNA polymerase. Transcriptional activator proteins in bacteria often operate by interaction with the C-terminal domain of the alpha-subunit of RNA polymerase (RNAP). In *B. subtilis* was discovered an "anti-alpha" factor Spx that blocks transcriptional activation by binding to the alpha-C-terminal domain, thereby interfering with the capacity of RNAP to respond to certain activator proteins. Spx disrupts complex formation between the response regulators ResD and ComA and promoter-bound RNAP, and it does so by direct interaction with the alpha-subunit. ResD- and ComA-stimulated transcription requires the proteolytic elimination of Spx by the ATP-dependent protease ClpXP. Spx represents a class of transcriptional regulators that inhibit activator-stimulated transcription by interaction with α-subunit of RNAP (Nakano et al., 2003).

As it can be seen there exist varieties of mechanisms which can influence the behaviour of a certain two-component system. Some of these mechanisms were demonstrated also for the DegS-DegU pair with respect to some target gene regulation, or concern directly the activity of DegS and DegU proteins. The compilation of different regulatory mechanisms is in a good agreement with the various cellular processes regulated by this two-component system and from here the wide range of target genes subjected to DegU regulation according to the requirements of the organism.

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VII. Appendix

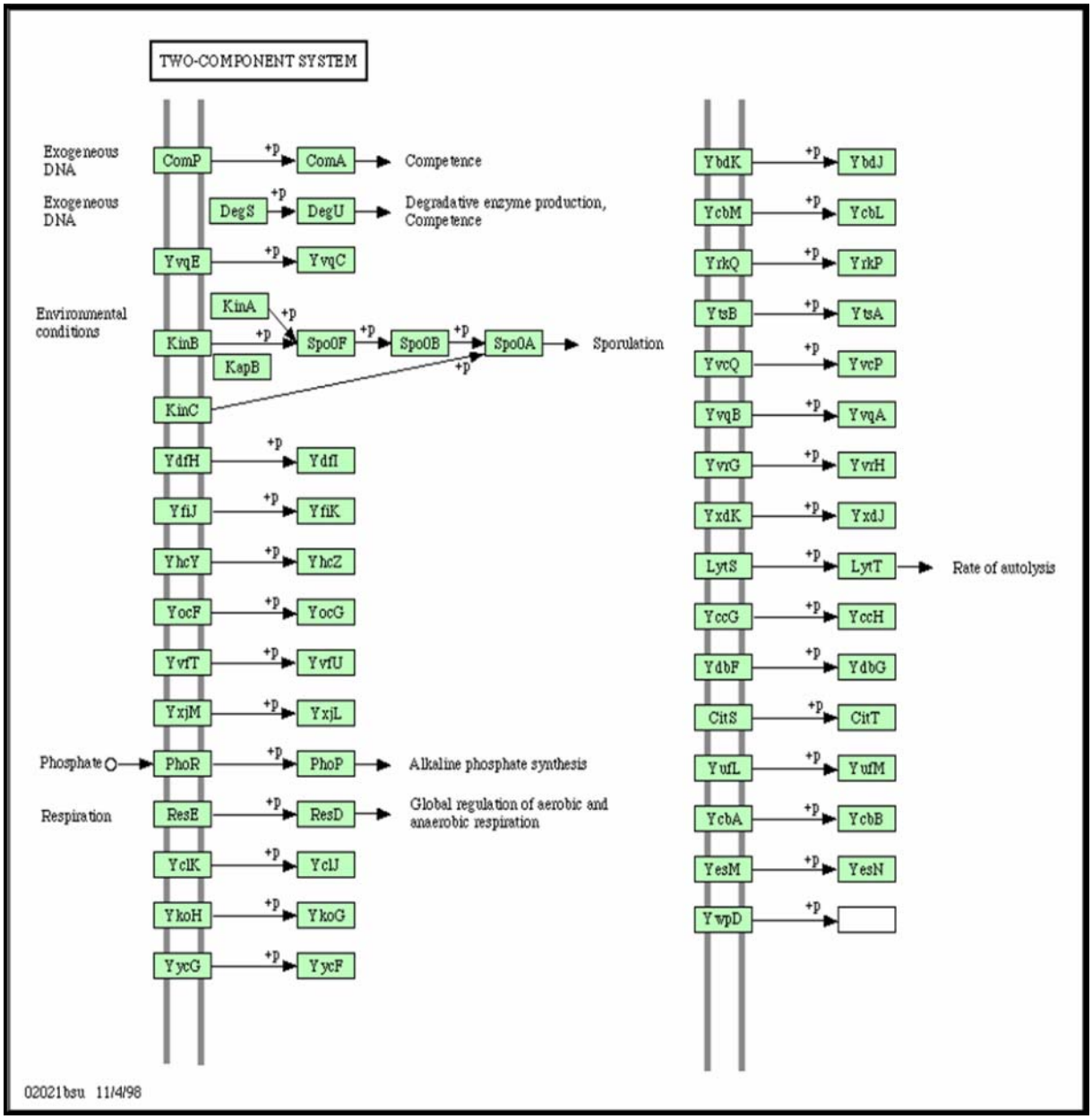
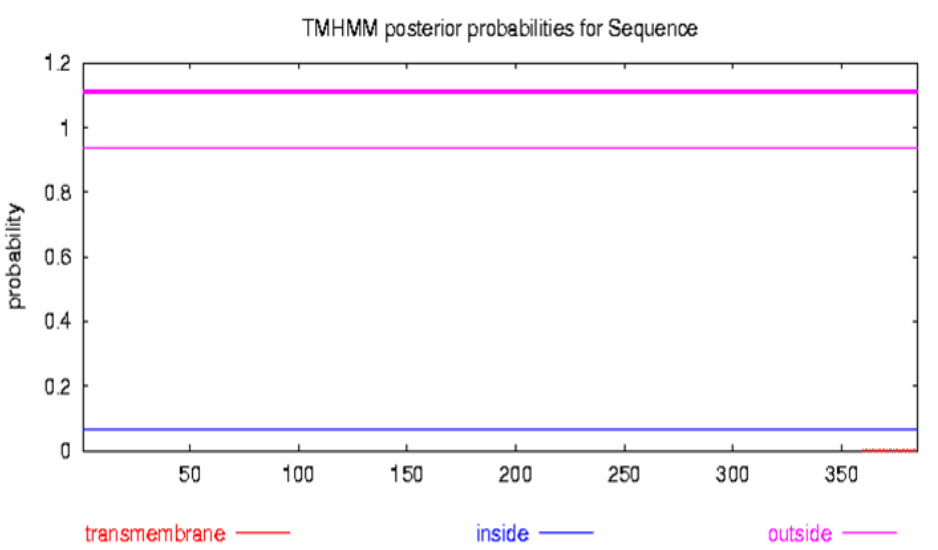


Fig. 39. Two-component signal transduction network in *B. subtilis*

In *B. subtilis* genome have been found 36 sensor kinases and 35 response regulators altogether (Fabrett et al., 1999; http://www.genome.jp/dbget). There is also one representative of the phosphorelay system pattern that comprises the signal network involved in sporulation process. Among the rest histidine kinase-response regulator pairs, only the DegS-DegU two-component system has cytoplasmic localized sensor kinase.

DegS



DegU

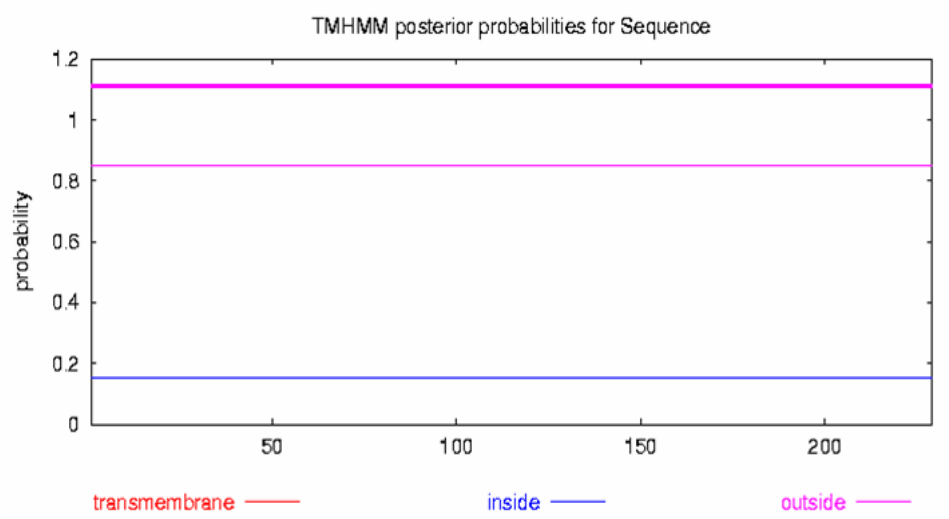


Fig. 40. DegS histidine kinase and DegU response regulator are cytoplasmic proteins

Both proteins appear to be located in the cytoplasm since they do not possess any hydrophobic domains within their amino acid sequences. The analysis is performed with the program TMHMM 2.0 (Transmembrane Protein Topology with a hidden Markov Model; http://www.cbs.dtu.dk/services/TMHMM-2.0)

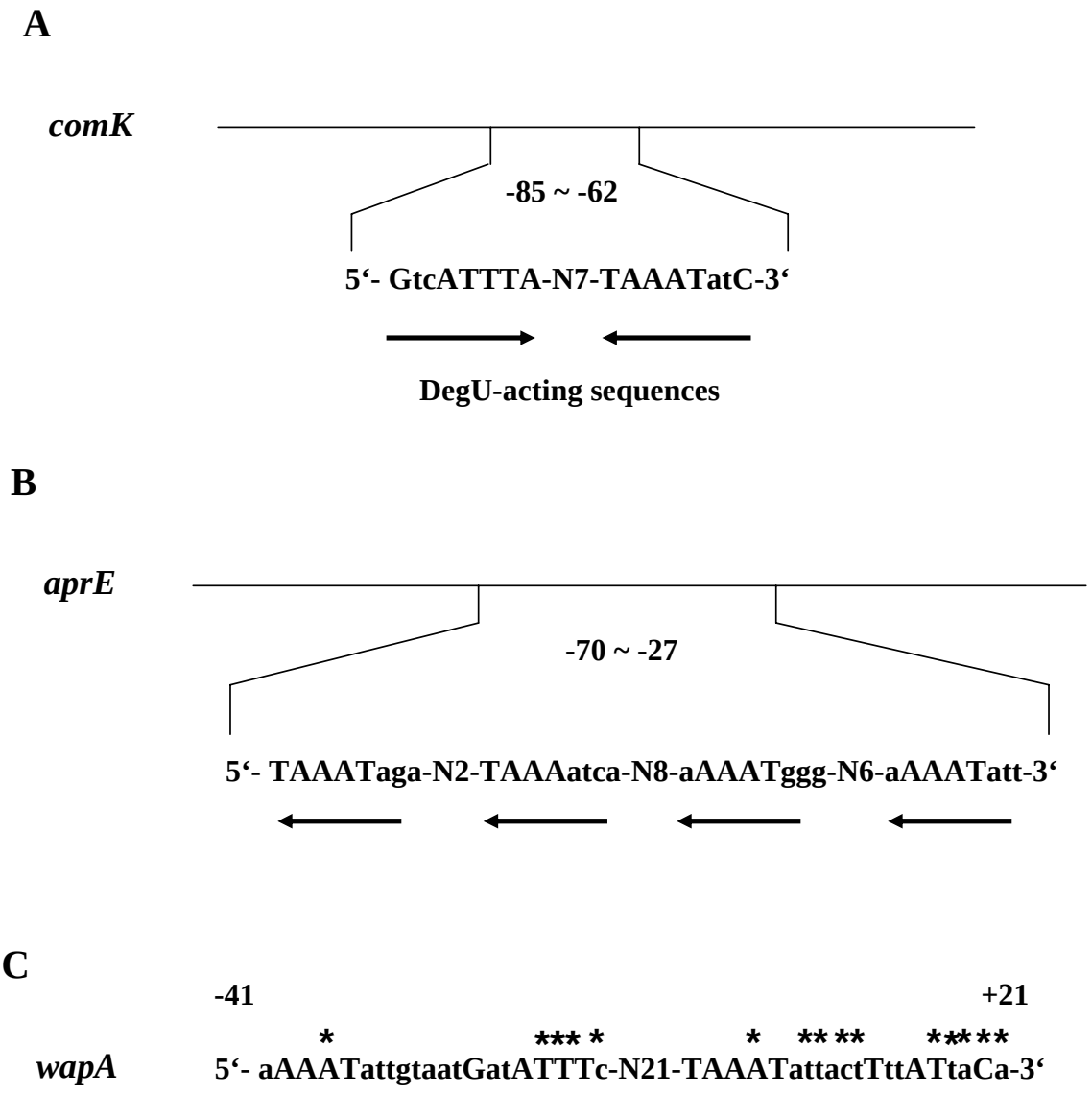


Fig. 41. Schematic representation of the cis-acting sequences for DegU

The numbers indicate the nucleotide positions relative to the transcription start site. Arrows show the cis-acting sequences for DegU. Nucleotides that are complementary to each other are indicated with uppercase. **A.** Shown is the *comK* promoter region (Shimane and Ogura, 2004). **B.** Part of the control region of *aprE* is present (Shimane and Ogura, 2004). **C.** The *wapA* promoter region is depicted. Asterix indicate the nucleotides whose substitution resulted in deregulation of phosphorylated DegU- dependent repression when the cells were grown under high salt conditions (Ogura et al., 2001).

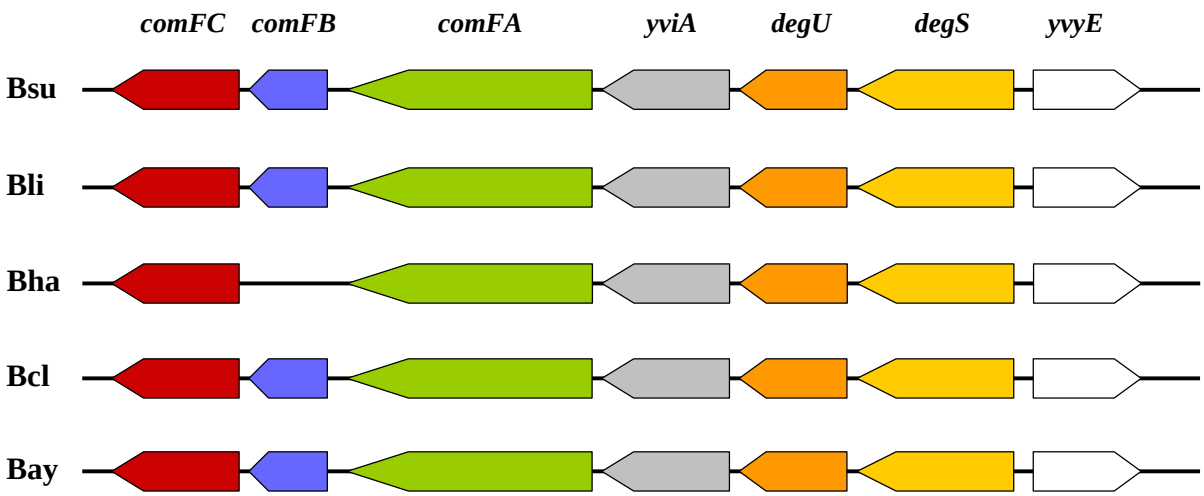


Fig. 42. Genomic map of the *degSU* locus in different *Bacillus* species

Shown is the preservation of the *degSU* genomic locus. Orthologous genes are indicated by identical colour. For the genomic localization of the presented genes the KEGG Genes Database is used (Kyoto Encyclopaedia of Genes and Genomes; http://www.genome.jp/kegg). Following organisms are applied: Bsu: *Bacillus subtilis*; Bli: *Bacillus licheniformis*; Bha: *Bacillus halodurans*; Bcl: *Bacillus clausii*; Bay: *Bacillus amyloliquefaciens*.

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Herewith I confirm that I wrote my dissertation

Transcriptional activation and sensing properties of DegS/DegU:
A two-component system involved in the osmotic regulation of *Bacillus subtilis*

without utilizing any illegitimate help. I used no other than the cited references and facilities, and this work has not been previously handed in another university.

Marburg/Lahn, November 2007 Teodora Hadzhieva

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